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Genética de la conservación en islas : el caso del alimoche canario

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GENÉTICA DE LA CONSERVACIÓN EN ISLAS

EL CASO DEL ALIMOCHE CANARIO

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A mis padres que siempre me transmitieron
un profundo respeto por la naturaleza

'Con respecto a la isla de Herbania, llamada Fuerteventura,
hay una especie de pájaros blancos y del tamaño de una oca,
que andan continuamente alrededor de la gente y
no dejan ninguna basura'

Le Canarien.

Crónicas francesas de la conquista de Canarias

Siglo XV

Abstract

Most of natural populations are seriously reduced and fragmented, including the insular ones, and they are susceptible to genetic erosion and its consequences (inbreeding depression). We have evidences showing that genetic erosion jeopardizes the future of wild populations, and therefore its studio, a priority in the current conservation biology, is necessary to take adequate measures to halt such deterioration. This thesis presents a particular case of an insular, reduced and threatened population that is subjected to genetic erosion; the Canarian Egyptian vulture. Our results show that reduced, insular populations present impoverished levels of genetic diversity, both at neutral and functional loci, in relation to their continental counterparts. We show furthermore, that drift is the dominant evolutionary force. However, we note that the potential existence of connection between these populations may have partly eased the impoverishment and therefore it has to be taken into account when establishing conservation measures. We have also observed that certain evolutionary mechanism, the co-evolution of the two copies of a functional gene (MHC), may have also been able to alleviate, to some extent, the loss of diversity suffered by island populations. The results of this research evidence, nevertheless, the existence of inbreeding depression manifested by a negative effect on the reproductive capabilities of individuals. Should be noted, finally, the recent formation of this population (some 2500 years or 200 generations, associated with the arrival of humans in the Canary Islands) that contrasts with the process of extinction that this deme seems to be suffering in the present. This fact seems to reflect the ephemeral and vulnerable nature of island populations. Present results indicate that loss of diversity negatively affects individuals and as a result, may compromise the survival of populations. It is therefore necessary to carry out genetic management measures in reduced and highly threatened populations, even when the levels of loss of diversity are not alarming.

Resumen

La mayoría de las poblaciones naturales se encuentran gravemente reducidas y fragmentadas, incluidas las insulares, y son susceptibles a la erosión genética y a sus consecuencias (depresión por endogamia). Contamos con evidencias que demuestran que la erosión genética compromete el futuro de las poblaciones silvestres y por eso su estudio es una prioridad en la actual biología de la conservación, necesario para poder tomar medidas que frenen dicho deterioro. La presente tesis plantea un caso concreto de población insular, reducida y amenazada sujeta a la erosión genética; el Alimoche canario. Nuestros resultados demuestran que las poblaciones insulares y reducidas presentan empobrecidos niveles de diversidad genética tanto neutral como funcional, en relación a sus equivalentes continentales, y que es la deriva la fuerza evolutiva dominante. Sin embargo, observamos que la potencial existencia de conexión entre dichas poblaciones puede aliviar en parte dicho empobrecimiento y por lo tanto ha de tenerse en cuenta a la hora de establecer medidas de conservación. Hemos observado además, que cierto mecanismo evolutivo, la co-evolución de las copias de un gen funcional (MHC), ha podido aliviar también, en cierta medida, la pérdida de diversidad sufrida por las poblaciones insulares. Los resultados de esta investigación evidencian, no obstante, la existencia de depresión por endogamia en la población insular estudiada, que se manifiesta en un efecto negativo sobre la capacidad reproductiva de los individuos. Cabe destacar, por último, la reciente formación de esta población (hace 2500 años o 200 generaciones, asociada a la llegada de los humanos a las islas Canarias) que contrasta con el proceso de extinción que parece estar sufriendo en el presente. Este dato parece reflejar el carácter efímero y vulnerable de las poblaciones insulares. Los resultados de este trabajo indican que la pérdida de diversidad afecta negativamente a los individuos y consecuentemente, puede comprometer la supervivencia de las poblaciones. Es necesario por lo tanto, realizar medidas de gestión genética en las poblaciones reducidas y altamente amenazadas, aún cuando los niveles de pérdida de diversidad no sean alarmantes.

Contents

List of Original Publications

I. Agudo R., Roques S., Galarza J.A., Rico C., Hiraldo F. & Donázar J.A. (2008). Isolation and characterization of 18 microsatellite loci in the Egyptian vulture (*Neophron percnopterus*). *Conservation Genetics*, 9, 1345-1348.

II. Agudo R., Rico C., Hiraldo F. & Donázar J.A. (2011). Evidence of connectivity between continental and differentiated insular populations in a highly mobile species. *Diversity and Distributions* 17, 1-10.

III. Agudo R., Alcaide M., Rico C., Lemus J.A., Blanco G., Hiraldo F. & Donázar J.A. (in press). MHC variation in insular populations of the Egyptian vulture: inferences about the roles of genetic drift and selection. *Molecular Ecology*.

IV. Agudo R., Rico C., Vilà C., Hiraldo F. & Donázar J.A. (2010). The role of humans in the diversification of a threatened island raptor. *BMC Evolutionary Biology* 10: 384.

V. Agudo R., Alcaide M., Rico C., Carrete M., Hiraldo F. & Donázar J.A. Genetic diversity at neutral and adaptive loci determines individual fitness in a long-lived territorial bird. *Manuscript*

General Introduction

Conservation Genetics, an emergency discipline for a World in Crisis

Charles Darwin would have never imagined that, only a few decades after starting to understand the mechanisms that create biodiversity, we would have to understand, imperatively, the mechanisms that destroy diversity on Earth in order to try to protect it. Even though the scientific and technological development are contributing extraordinary to the understanding of the processes that generate and maintain diversity, we barely now what happens at the top of a huge iceberg that melts very rapidly.

The great 'sixth extinction', as it has been named, is the dramatic process of extermination that the species are suffering. This extinction is directly related to human action (Leakey & Lewin, 1995). The 'conservation biology' discipline emerged in the 80th (Soulé 1985) as a response to this environmental crisis and in the search for tools to preserve biodiversity. It has been appropriately described as a 'crisis discipline'. The magnitude of such crisis is manifested in the number of species that are currently facing a problem of imminent extinction (some of the most pessimistic estimates suggest that between 15 and 20% of the species became extinct between 1980 and 2000 (World Conservation Monitoring Centre (WCMC) 1992)). The fact that

conservationist have to take quick decisions based on the limited information available highlights also the urgency complexion of this discipline.

The main deterministic factors that promote the extinction of the species are loss of habitat, introduction of exotic species, over-exploitation and pollution. These factors lead to the reduction of population sizes and this in turn, exposes populations to stochastic factors; environmental, demographic or genetic (WCMC 1992). Thus, although the factors that caused the populations decline are eliminated, problems associated with a reduced population size will persist (Frankham 2003). Small populations are vulnerable to genetic drift and therefore exposed to the loss of genetic diversity. Populations with low variability suffer from a diminished capacity of response to the environmental changes and hence a reduced adaptive potential (Charlesworth & Charlesworth 1987; England *et al.* 2003; Swindell & Bouzat 2005). In addition, small populations have a greater likelihood that mating occur between relatives which often leads to an increase of inbreeding. Inbreeding may reduce individual fitness and survival (by inbreeding depression) accelerating the population extinction risks (Madsen *et al.* 1996, Lacy 1997, Acevedo-Whitehouse *et al.* 2003, Liberg *et al.* 2005, Vilas *et al.* 2006). This process is known as 'genetic erosion' and constitutes an element of primary importance in the study of isolated populations (Mills & Allendorf 1996).

The information collected during the last decade indicates that, at present, many wild populations are severely reduced and fragmented, and are unavoidably, subjects of the genetic erosion (Crnokrak & Roff 1999; Aguilar *et al.*, 2008; Frankham 2010a). Despite the controversy that existed until recently about the contribution of inbreeding on the population's risk of extinction (Lande, 1988; Caro & Laurenson, 1994; Caughley, 1994; Dobson,

1999), we have now enough evidences, both theoretical and empirical, showing that inbreeding undertake considerably the future survival of wild populations (Frankham 2005;) O'Grady *et al.* 2006). These facts set the genetic studies as a priority within the field of conservation biology.

The field of conservation genetics deals with the genetic factors that are affecting the risk of extinction of populations and aim to develop appropriate management plans to minimize these risks. Its main tool is the use of molecular polymorphic markers. Technological advances obtained in the field of the molecular biology (since the invention of the PCR (polymerase chain reaction) in 1988 (Saiki *et al.* 1988)), have allowed us to address questions that were previously inaccessible with the traditional techniques. Today, we can determine the genetic diversity, connectivity and the degree of differentiation between isolated populations, as well as detecting population declines or bottlenecks and calculating effective population sizes. The genetic study of populations also allows setting the degree of kinship between individuals, the levels of inbreeding and the existence of inbreeding depression. This information is essential for: a) defining management units and populations of concern (i.e. differentiated and threatened populations), b) resolving taxonomic uncertainties, c) detecting losses of genetic diversity within populations, d) assessing the existence of connectivity between them and e) estimating their effective population size. Potential solutions to alleviate the problems associated with the loss of genetic diversity, such as introductions and translocations, may arise from this information. In addition, knowing the degree of kinship between individuals is essential for a proper management of the breeding programs in captivity and reintroduction.

There are several examples where it was shown how the introduction, establishment and reproduction of individuals (outcrossing) in severely reduced populations with high levels of inbreeding, greatly improved their viability (Vrijenhoek, 1994; Westemeier *et al.*, 1998; Madsen *et al.*, 1999; Ebert *et al.*, 2002; Vila *et al.*, 2003; Schwartz and Mills, 2005; Pimm 2006; Hedrick & Fredrickson 2008). Similarly, the genetic erosion that many populations are currently suffering due to the fragmentation of their habitats and the reduction of their sizes, would be alleviated through a proper management that promoted gene flow between them (Frankham 2010b). However, and whereas the captive-breeding programmes do employ, in most cases, the molecular techniques and the consequent genetic information, their application in the management of endangered wild populations is still in its infancy (Husband & Campbell, 2004; Frankham 2010a).

Several reasons could explain, from my point of view, the recurrent absence of genetics in the conservation plans to date. On one hand, molecular genetics is a relatively young discipline (less than half a century) and fairly unknown to many researchers working in conservation biology. Furthermore, its implementation has been traditionally quite expensive and therefore, inaccessible in many cases. Nonetheless, recent technological developments and lower costs are enabling a more widespread use of molecular tools. On the other hand, there has been some scepticism about the importance of the role of genetics in the conservation of populations and its application has been only considered in a few examples such as the ones mentioned above, and others emblematic cases of severe inbreeding depression (e.g. the Florida Panther (*Puma concolor couguar*) or the California Condor (*Gymnogyps californianus*) among others (Pimm 2006; Hedrick & Fredrickson 2008)). Finally, there is a widespread fear about the consequences of the outbreeding depression (i.e. fitness reduction of loss due to the cross-breeding with other distinct populations).

This fear hampers the implementation of rational genetic management of fragmented populations (Frankham 2010a). However, there is little empirical evidence of this phenomenon and it is thought that its risks have been exaggerated (Frankham 2010b). There is though, need of more scientific effort to understand and predict these risks.

Therefore, one of the main issues that has to be addressed, as a priority, within the field of conservation genetics (which, furthermore, justifies the development of the present thesis) is the understanding of the role of the genetic factors in the decline and risk of extinction of wildlife populations. To address this question it is necessary to properly integrate the genetic information with a comprehensive individual ecological knowledge, as it is the case here.

Islands and long-lived vertebrate species

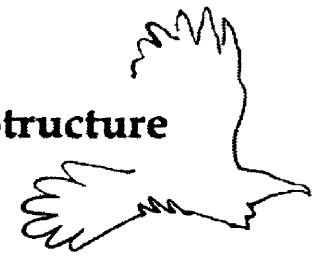
Oceanic islands (i.e. those that were formed on the marine platforms and have never been connected to the mainland) are characterized by their wealth of endemic species, due to the evolutionary processes that result from isolation, but also and unfortunately, by their high rates of threat and extinction of their populations and species. The majority of extinctions documented since 1600 correspond to island species even though they represent a small proportion on Earth's biodiversity: only 20% of birds inhabit islands, but 80% of extinct species to date were insular (Myers 1979). The reasons for the increased vulnerability of insular populations respect to their continental counterparts are still controversial. It has been highlighted their greater sensitivity to the stochastic factors, both demographic and environmental

(Pimm 1991). This susceptibility can, however, be predicted in genetic terms (Frankham 1998). Island populations are naturally small and have gone through a bottleneck during their foundation. This determines the inevitable loss of genetic diversity and the consequent increase of inbreeding resulting from a reduced and finite population size (Frankham 1998). Frankham demonstrated in two comparative studies (Frankham 1997, 1998) that insular populations have increased inbreeding values and significant losses of genetic diversity compared to continental populations, explaining thus their greater risk of extinction. This vulnerability determines that the impact of human activity on diversity is more dramatic in the islands than in continental biomes. Thus, most of extinctions occurred on islands are directly related to the colonization of humans. It is evidenced by, for example, the mass extinctions occurred in the Pacific islands or in the Mediterranean Sea after the arrival of humans (Steadman 2006; Alcover *et al.* 1998, Bover & Alcover 2008).

Whereas it is clear that human action has accelerated extinction processes, the fact that the island fauna is currently represented by a small proportion of what existed in the past (Alcover & McMinn 1994), could be indicative of the natural character of vulnerability of insular populations. In particular, long-lived species are less represented in the islands due to, in part, their larger sizes and consequently, their increased ecological requirements (Whittaker & Fernández-Palacios 2006). Furthermore, long-lived species present higher risks of extinction because of their characteristically conservative life strategy (i.e. low birth rates and high adult survival) (Newton 1979; Alcover & McMinn 1994; White & Kiff 2000; Donázar *et al.* 2005). This intrinsic vulnerability can be determining, to a greater or lesser extent, the failure that many management programs have had when aiming to preserve long-lived species on islands.

The conservation of long-lived species, insular or continental, is very important because they can act as umbrella species (i.e. their preservation enables the parallel conservation of other threatened species with lower social charisma) (Sergio *et al.* 2006). However, their study and conservation is difficult since obtaining individualized information necessary for a proper management involves long-term monitoring. The combination of ecological monitoring with the molecular tools can alleviate these difficulties. Apart from being essential to assess the levels of genetic diversity, molecular techniques allow, for example, the identification of individuals (both previously captured or by using noninvasive sampling as feathers), the determination of the degree of kinship between individuals and the individual level of inbreeding without the necessity of constructing pedigrees (which would imply several decades of monitoring).

Goals and Thesis Structure



The main objective of this thesis is to analyse the role of genetic factors in the permanence of insular populations of long-lived species. Our study model is conformed by the two main insular populations (Canary and Balearic Islands) and the main continental deme (Iberian Peninsula) of the Egyptian vulture (*Neophron percnopterus*) in the Western Palearctic. This choice is based on the following reasons: 1) the Egyptian vulture is one of the most globally endangered raptor species (BirdLife International 2008), 2) it presents both continental as island populations which makes it possible to test hypotheses based on comparative approaches, 3) the Canarian Egyptian vulture population (main subject of this thesis) has been carefully monitored over the last decade and as a result, it has been able to establish what are the environmental and ecological mechanisms that are determining its decline.

The two main hypothesis that we wanted to test can be summarized as follows: i) island populations present lower values of both neutral and adaptive genetic diversity than their continental counterparts and ii) this loss of genetic diversity has an important effect on individual fitness (what would consequently may affect the viability of the population).

To test these hypotheses we employ neutral polymorphic markers (microsatellites) and we incorporate functional loci, specifically genes of the Major Histocompatibility Complex (MHC). From the international scientific community it is

increasingly recommended the use of functional genes in the genetic studies focused on the species conservation (Vernesi *et al.* 2008; Frankham 2010a, 2010b). The main reason is the necessity for seeking direct relationships between genetic diversity and the parameters related to the individual fitness. In addition, several studies have highlighted the lack of correlation between neutral and adaptive genetic diversity as well as between neutral diversity and the individual ecological parameters (Coltman & Slate 2003; Väli *et al.* 2008). Lack of relationship as well as the existence of random correlations can lead to erroneous conclusions and have an impact on the success of the management of populations.

The thesis is structured into five independent but closely interlinked chapters. **Chapter 1** describes the isolation and characterization of the neutral molecular markers (microsatellites) specifically designed for the development of this thesis. Addressing complex objectives require having the right tools. The use of specific markers is recommended, especially in the case of genetic studies of threatened populations (Primmer *et al.* 1996). Heterospecific markers often show decreased levels of polymorphism when applied to species different to those from which they were originally designed (referred to as 'ascertainment bias' (Ellegren 1995)). Furthermore, the development of the last part of this thesis (analysis of kinship and degree of inbreeding in the Canarian Egyptian vultures) required a large panel of polymorphic markers. For all these reasons our first aim was to develop at least 20 specific markers that allowed us to address the objectives of the present thesis.

The main objective of **chapters 2 and 3** is to analyze the relationship between insularity and genetic diversity. We do this by carrying out a comparative study between continental and insular populations of the Egyptian vulture in the Western

Palearctic. We investigate the genetic diversity, structure and gene flow between them.

Chapter 2 deals with the study of the population genetics by analysing the diversity of neutral markers (microsatellites). A primary objective within the conservation genetics is to identify the units of management. It is then essential to determine the levels of differentiation and gene flow between populations. This information is especially relevant for insular populations which are more vulnerable to the effects associated with small population sizes, such as genetic drift and the consequent loss of gene diversity. Insular populations are traditionally considered as separate conservation units, due to their characteristic isolation and differentiation. However, island populations of highly mobile species may receive migrant mainland individuals. This immigration may buffer the effects of demographic stochasticity and genetic drift and thus reduce the risk associated with inbreeding depression (Westemeier *et al.* 1998, Madsen *et al.* 1999; Frankham, 2002; Marr *et al.* 2002; Vilà *et al.* 2003; Hogg *et al.* 2006). The main objective of this chapter is then to test if oceanic populations can be connected to their continental counterparts and to discuss the consequences in terms of conservation.

In **Chapter 3** we incorporate the study of functional genes to analyse again the genetic diversity and differentiation in the Western Palearctic populations of Egyptian vulture. Whereas neutral genetic variation is very useful to test differentiation and gene flow, it provides a fairly vague indication of the evolutionary potential of populations (Balloux *et al.* 2004; Slate *et al.* 2004; Hansson & Westerberg 2008; Chapman *et al.* 2009). On the contrary, the study of functional genes can provide us more precise information about the distribution of adaptive genetic variation and the possible negative effects related to the loss of genetic diversity (Hedrick 2000). On the other hand, the

comparative study between neutral and functional genetic variability, as well as between populations subjected to different selective pressures, can help to understand the evolutionary mechanism drawing the genetic pattern of populations. This is one of the main objectives of molecular ecology and evolutionary biology. Though, very few functional genes have though been characterised in natural populations. In this regard, genes of the major histocompatibility complex (MHC) are an exception and have become ideal candidates for this type of studies. First, they are among the best studied functional genes in the animal world. Second, they constitute an essential component of the immune system of individuals, which is directly related to individual fitness and survival (Oliver *et al.* 2009, Radwan *et al.* 2009; Spurgin & Richardson 2010). The main objective of this chapter is to determine the genetic differences (both quantitative and qualitatively) in functional genes among insular populations (small and genetically impoverished) and their continental equivalents. We asses this goal by studying the genetic variability in the genes of the MHC class II β. This information is intended to elucidate the implications of such differences in terms of conservation, and to discuss the possible selective forces that have drawn the observed pattern.

In **chapter 4** we analyse and date the process of colonization and differentiation of an insular population of a long-lived vertebrate (the canarian Egyptian vulture). The use of molecular tools and recent analytical techniques allow us to infer past demographic events. In this chapter and based on the examination of the neutral genetic diversity using Bayesian analyses, we disentangle how and when could the insular population have arose. Furthermore, we discuss the role of humans in such colonization and differentiation.

In the last **chapter (5)**, we raise the scale of study and pass from the population to the individual level. The next step after clarifying the previous issues (which are the units of management?, which are the levels of neutral and functional genetic diversity?, is there a genetic problem at population level?, is there gene flow among populations?, can such genetic problems be alleviated?) is to clarify what are the consequences of the loss of genetic diversity in the populations, i.e. the existence of inbreeding depression and its effects. To address this question is necessary a deep knowledge at both ecological and genetic level, of the populations. Unfortunately, most studies do not reach this degree of understanding due to the lack of individual information, especially in the case of long-lived species. One of the most important contributions of this thesis is therefore, the determination of the effects of inbreeding on the individual fitness of an insular and threatened population of a long-lived vertebrate species. On the other hand, the majority of existing studies that have analyzed inbreeding depression are based on indirect estimates of inbreeding using neutral markers (such as the individual heterozigosidad). However, the extent to which heterozygosity at a few neutral loci reflects genome-wide diversity remains controversial (Balloux *et al.* 2004; Slate *et al.* 2004; Hansson & Westerberg 2008; Väli *et al.* 2008; Chapman *et al.* 2009). For this reason it is highly recommended the parallel scan of genes with evolutionary significance. Unfortunately, few functional genes have been characterised in wild species and are hence available for their use in natural populations. In this regard, as it has already been described for Chapter 3, MHC genes are the exception. The use of these genes in the research of inbreeding depression makes sense since they are directly related to immune capacity of individuals, and therefore with their fitness and survival (Oliver *et al.* 2009, Radwan *et al.* 2009; Spurgin & Richardson 2010). That is why we incorporate the study of these functional and evolutionarily significant genes in the individual

genetic study of the Canarian Egyptian vulture population. We believe that this fact makes this thesis to be an important contribution in the field of genetics and the conservation biology of vertebrates.

Introduction General

Genética de la Conservación, una disciplina de urgencia para un mundo en crisis

Darwin jamás habría podido imaginar que tan sólo unas cuantas décadas después de empezar a comprender los mecanismos que han generado la diversidad de la vida en la Tierra, tendríamos que estudiar con urgencia y a contrarreloj, cómo operan los mecanismos que la destruyen para así intentar protegerla. Aunque el desarrollo tecnológico y científico esta contribuyendo de forma extraordinaria al entendimiento de los procesos que generan y mantienen la biodiversidad, apenas comprendemos hoy lo que ocurre en la punta de un enorme iceberg que se derrite a pasos agigantados.

La gran 'sexta extinción' es cómo se le ha denominado al proceso dramático de desaparición que están sufriendo las especies y que está directamente relacionado con la acción humana (Leakey & Lewin, 1995). En respuesta a esta crisis ambiental y en una búsqueda de las herramientas necesarias para preservar la biodiversidad, surge en la década de los 80 la biología de la conservación (Soulé, 1985), descrita con acierto como una 'disciplina de crisis'. La magnitud de la crisis objeto de esta disciplina queda reflejada en el número de especies que actualmente se enfrentan a un problema de extinción inminente (algunas de las estimas más pesimistas indican que entre el 15 y el 20% de las especies se extinguieron entre 1980 y 2000 (World Conservation Monitoring Centre (WCMC) 1992)). El hecho de que los científicos conservacionistas deban tomar decisiones rápidas

basadas en la escasa información disponible, resalta también el cariz de urgencia de esta disciplina.

Los principales factores determinísticos que provocan la extinción de las especies son la pérdida de hábitat, la introducción de especies exóticas, la sobreexplotación y la contaminación. Estos factores provocan la reducción de los tamaños poblacionales y ésta a su vez, expone a las poblaciones a factores estocásticos; ambientales, demográficos o genéticos (WCMC 1992). De este modo, aunque los factores que originaron el declive de las poblaciones sean eliminados, los problemas asociados a un reducido tamaño de población persisten (Frankham, 2003). Las poblaciones pequeñas, vulnerables a la deriva génica, se encuentran expuestas a la pérdida de diversidad genética. Las poblaciones con poca variabilidad ven reducida su capacidad de respuesta a los cambios ambientales y consecuentemente, su potencial adaptativo (Charlesworth & Charlesworth, 1987; England *et al.* 2003; Swindell & Bouzat, 2005). Además, las poblaciones reducidas presentan una mayor probabilidad de que se produzcan apareamientos entre relativos lo que frecuentemente conlleva a un aumento de la endogamia y los riesgos asociados, como la reducción de la productividad y la supervivencia (depresión por endogamia), acelerando así su riesgo de extinción (Madsen *et al.*, 1996; Lacy & Horner, 1987; Acevedo-Whitehouse *et al.*, 2003; Liberg *et al.*, 2005; Vilas *et al.*, 2006). Este proceso es conocido como erosión genética y constituye un elemento de primera importancia en el estudio de las poblaciones aisladas (Mills & Allendorf, 1996).

La información recopilada durante la última década indica que actualmente, existen muchas poblaciones en el planeta que se encuentran gravemente reducidas y fragmentadas, lo que las convierte inevitablemente en objeto de la erosión genética (Crnokrak & Roff 1999; Aguilar *et al.*, 2008; Frankham 2010a). Por

otro lado y a pesar de la controversia que existía hasta hace poco acerca de la contribución de la endogamia en el riesgo de extinción (Lande, 1988; Caro & Laurenson, 1994; Caughley, 1994), contamos hoy con suficientes evidencias tanto teóricas como empíricas que demuestran que la endogamia y la consecuente depresión por endogamia comprometen muy sustancialmente el futuro de las poblaciones silvestres (Frankham 2005; O'Grady *et al.* 2006). Esos hechos han situado a los estudios genéticos en un lugar prioritario dentro de la biología de la conservación.

La genética de la conservación se ocupa pues del estudio de los factores genéticos que afectan al riesgo de extinción de las poblaciones, como base para la elaboración de planes de gestión adecuados que minimicen dichos riesgos. Su herramienta principal es el uso de marcadores polimórficos moleculares. Los avances tecnológicos conseguidos en el campo de la biología molecular, desde la invención de la PCR (o reacción en cadena de la polimerasa) en 1988 (Saiki *et al.* 1988) han permitido abordar cuestiones antes inaccesibles con las técnicas tradicionales. Hoy día, podemos determinar la diversidad genética, la conectividad y el grado de diferenciación que existe entre poblaciones aisladas, así como detectar declives poblacionales o cuellos de botella y calcular los tamaños efectivos. El estudio genético de las poblaciones permite además establecer el grado de parentesco entre los individuos, los niveles de endogamia y la existencia de depresión por endogamia. Esta información es esencial para: a) definir las unidades susceptibles de gestión y protección (es decir poblaciones diferenciadas y/o amenazadas), b) resolver dudas taxonómicas, d) detectar pérdidas de diversidad genética en las poblaciones y e) evaluar la existencia de conectividad entre ellas. A partir de esta información se pueden plantear soluciones que permitan aliviar los problemas asociados a la pérdida de diversidad genética, como son las translocaciones o las introducciones. Además, conocer el grado de parentesco entre los

individuos es esencial para una adecuada gestión de los programas de cría en cautividad y reintroducción.

Existen varios ejemplos sobre cómo la introducción de individuos y su establecimiento y reproducción ('outcrossing') en poblaciones severamente reducidas y con elevados niveles de endogamia, mejoró considerablemente la viabilidad de dichas poblaciones (Vrijenhoek, 1994; Westemeier *et al.*, 1998; Madsen *et al.*, 1999; Ebert *et al.*, 2002; Vila *et al.*, 2003; Schwartz & Mills, 2005; Pimm 2006; Hedrick & Fredrickson 2008). Del mismo modo, la erosión genética que muchas poblaciones sufren actualmente debido a la fragmentación de su hábitat y la reducción de sus tamaños, se aliviaría mediante una gestión adecuada que promoviera el aumento del flujo génico entre ellas (Frankham 2010b). Sin embargo y mientras que los programas de cría en cautividad emplean, en la mayoría de los casos, técnicas moleculares y la información genética obtenida a través de ellas, su aplicación en la gestión de las poblaciones silvestres amenazadas está aún en sus albores (Husband & Campbell, 2004; Frankham 2010a).

Varios son los motivos que, desde mi punto de vista, podrían explicar la recurrente ausencia de la genética en los planes de conservación de las poblaciones amenazadas. Por un lado, la genética molecular es una disciplina relativamente joven (menos de medio siglo) y en cierto modo algo ajena a muchos investigadores que trabajan en el campo de la biología de la conservación. Además, su aplicación ha sido tradicionalmente costosa y por lo tanto inaccesible en muchos casos. No obstante, el reciente desarrollo tecnológico y abaratamiento de los costes, están permitiendo un uso más generalizado de las herramientas moleculares. Por otro lado, existía cierto escepticismo entre los investigadores, acerca de la importancia del papel de la genética en la conservación de las poblaciones y su consideración se ha

visto reducida a los escasos ejemplos arriba mencionados, o a los casos extremos de depresión por endogamia como son, por ejemplo, la pantera de Florida (*Puma concolor couguar*) o el cóndor de California (*Gymnogyps californianus*) (Pimm 2006; Hedrick & Fredrickson 2008). Por último, existe un temor generalizado a las consecuencias de la depresión por entrecruzamiento (o 'outbreeding depression'), es decir, a que las poblaciones pierdan eficacia biológica como resultado del entrecruzamiento con otras poblaciones distintas. Este temor puede obstaculizar la implementación de una gestión genética racional de las poblaciones fragmentadas (Frankham 2010a), más cuando apenas existen evidencias empíricas que demuestren ese fenómeno, cuyos riesgos se creen han sido exagerados (Frankham 2010b). Es cierto, no obstante, que aún es necesario un mayor esfuerzo científico para conocer y predecir dichos riesgos.

Una de las principales cuestiones a abordar de forma prioritaria dentro del campo de la genética y la biología de la conservación, la cual justifica además el desarrollo de la presente tesis, es de qué manera están contribuyendo los factores genéticos, respecto a otros factores, al declive y riesgo de extinción de las poblaciones silvestres. Para contestar a esa cuestión es necesario el desarrollo de un mayor número de estudios que integren adecuadamente la información genética con un conocimiento exhaustivo de la ecología de las poblaciones.

Las islas y los vertebrados de larga vida

Las islas oceánicas, es decir aquellas que se han formado sobre las plataformas marinas y nunca han estado conectadas al continente, se caracterizan por su riqueza en endemismos, debido a los procesos evolutivos resultado del aislamiento, pero también y desgraciadamente, por las elevadas tasas de amenaza y extinción de sus poblaciones y especies. La mayoría de las extinciones documentadas desde 1600 se corresponden con

especies insulares a pesar de que éstas son una minoría dentro del conjunto de las especies en la Tierra: solo el 20% de las aves habitan islas, sin embargo el 80% de las especies extintas hasta el momento eran insulares (Myers 1979). Las razones de la mayor vulnerabilidad de las poblaciones insulares respecto a sus equivalentes continentales son aún controvertidas, pero se ha resaltado su mayor sensibilidad a los factores de tipo estocástico, ya sean demográficos o ambientales (Pimm 1991). Esa susceptibilidad se puede, no obstante, predecir en términos genéticos (Frankham 1998). Las poblaciones insulares se encuentran naturalmente reducidas y han pasado por un cuello de botella durante su fundación, lo que ha determinado la inevitable pérdida de diversidad genética y el consecuente aumento de la endogamia como resultado de un tamaño poblacional finito y reducido (Frankham 1998). En dos estudios comparativos Frankham (1997, 1998) demostró que las poblaciones insulares presentan valores de endogamia mayores y pérdidas significativas de diversidad genética respecto a las poblaciones continentales, lo que explicaría su mayor riesgo de extinción. Esta vulnerabilidad ha determinado que el impacto de la acción humana sobre la diversidad haya sido y es aún hoy, más dramático en las islas que en los biomas continentales. Así, la mayoría de las extinciones acaecidas en islas están directamente relacionadas con la colonización de los humanos. Basta con, por ejemplo, revisar las extinciones masivas en las islas tropicales del Pacífico o las ocurridas en el mar Mediterráneo tras la llegada de los humanos (Steadman 2006; Alcover *et al.* 1998, Bover & Alcover 2008).

Sin embargo, aunque está claro que la acción humana ha acelerado los procesos de extinción, el hecho de que la fauna insular esté actualmente representada por una pequeña proporción de lo que existió en el pasado (Alcover & McMinn 1994), podría ser indicativo del carácter natural de vulnerabilidad de las poblaciones insulares. Particularmente, las especies de larga

vida, menos representadas en las islas debido en parte a su mayor tamaño y consecuente mayores requerimientos ecológicos (Whittaker & Fernández-Palacios 2006), presentan además mayores riesgos de extinción dada su estrategia de vida característicamente conservadora (Newton 1979; Alcover & McMinn 1994; White & Kiff 2000; Donázar *et al.* 2005). Esta mayor vulnerabilidad intrínseca puede determinar también, en mayor o menor medida, el escaso éxito que, en muchas ocasiones, tienen los programas de conservación de estas especies en islas.

La conservación de las especies de larga vida, ya sean insulares o continentales, es muy importante debido a que pueden actúar como especies paraguas (es decir, su conservación permite la conservación paralela de otras muchas especies amenazadas pero que cuentan con menor carisma social) (Sergio *et al.* 2006). Sin embargo, su estudio y conservación es difícil puesto que la obtención de la información individualizada y de calidad necesaria para una gestión adecuada, implica el seguimiento de los efectivos a largo plazo. Las técnicas moleculares, además de ser imprescindibles para evaluar los niveles de diversidad genética, pueden, en combinación con el seguimiento ecológico, aliviar en gran medida esa dificultad. Así permiten, por ejemplo, la identificación de los individuos (tanto previamente capturados como de forma indirecta mediante muestreos no invasivos como plumas, pelo o heces), la determinación del grado de parentesco entre individuos y la endogamia individual, sin necesidad de la construcción de pedigríes (lo que implicaría varias décadas de seguimiento).

Objetivos y Organización de la Tesis

El objetivo principal de la presente tesis es analizar el papel de los factores genéticos en la permanencia de las poblaciones insulares de vertebrados de larga vida. Como objeto de estudio hemos elegido las dos principales poblaciones insulares (Canarias y Baleares) y la principal población continental (Península Ibérica) de alimoche (*Neophron percnopterus*) que sobreviven en el Paleártico occidental. Esta elección se basa en que: 1) se trata de una de las especies de rapaces más amenazadas a nivel mundial (BirdLife International 2008), 2) presenta tanto poblaciones continentales como insulares por lo que es posible establecer hipótesis basadas en aproximaciones comparativas, 3) la población canaria, principal objeto de estudio de esta tesis, ha sido minuciosamente estudiada durante la última década y se han podido establecer cuales son los mecanismos ecológicos y ambientales que están determinando su declive.

La dos principales hipótesis que manejamos se pueden resumir en que: 1) las poblaciones insulares presentan niveles de diversidad genética inferiores, tanto neutral como adaptativa, respecto a sus equivalentes continentales y 2) esta pérdida de diversidad genética tiene un efecto relevante sobre la eficacia biológica individual (lo que afectaría consecuentemente a la viabilidad de la población).

Para testar estas hipótesis empleamos por un lado marcadores neutrales polimórficos (microsatélites) y además incorporamos marcadores funcionales, concretamente los genes

que se engloban dentro del Complejo Mayor de Histocompatibilidad (MHC). Desde el entorno científico internacional se recomienda cada vez más el empleo de genes funcionales en los estudios genéticos enfocados a la conservación de las especies (Vernesi *et al.* 2008; Frankham 2010a, 2010b). La principal razón es la búsqueda de relaciones directas entre diversidad genética y los parámetros que miden la salud y la supervivencia de los individuos, mucho más informativas en el caso de genes funcionales que en relación a marcadores neutrales. Además, varios estudios han resaltado la falta de correlación entre diversidad genética neutral y adaptativa, así como entre diversidad neutral y los parámetros ecológicos individuales (Coltman & Slate 2003; Väli *et al.* 2008). Tanto la falta de relación como la existencia de correlaciones azarosas, pueden acarrear conclusiones erróneas y repercutir en el éxito en la gestión de las poblaciones.

La tesis está estructurada en cinco capítulos que se presentan de manera independientes pero que guardan estrecha relación entre sí. En el **capítulo 1** describimos el aislamiento y caracterización de los marcadores moleculares neutrales (microsatélites) específicos diseñados para el desarrollo del presente trabajo. Antes de abordar objetivos más complejos era necesario contar con las herramientas adecuadas. El uso de marcadores específicos es recomendable, especialmente cuando se trata de obtener estimas de variabilidad genética en poblaciones amenazadas (Primmer *et al.* 1996) ya que los marcadores heteroespecíficos suelen presentar disminuidos niveles de polimorfismo cuando se aplican a otras especies distintas de la que se ha empleado para su diseño, lo que se denomina como 'ascertainment bias' (Ellegren 1995). Además, para el desarrollo de la última parte de esta tesis (análisis de parentesco y grado de endogamia en la población canaria de Alimoche) necesitábamos un gran número de marcadores polimórficos. Por todo ello

nuestro primer propósito fue desarrollar al menos 20 marcadores específicos que nos permitieran abordar los objetivos planteados.

El objetivo principal de los **capítulos 2 y 3** es analizar cual es la relación entre insularidad y diversidad genética. Para ello se realiza el estudio comparativo entre las poblaciones continentales e insulares del alimoche en el Paleártico Occidental y se analiza la diversidad, estructura y flujo génico entre ellas. El **capítulo 2** aborda el estudio de la genética poblacional a partir del análisis de la diversidad de los marcadores neutrales (microsatélites). Un objetivo primordial de la genética de la conservación es identificar cuales son las unidades de gestión. Para ello es imprescindible determinar la existencia de flujo entre poblaciones así como los niveles de diferenciación genética. Este análisis es especialmente relevante cuando se trata de poblaciones insulares (oceánicas) más vulnerables a los efectos asociados a los tamaños reducidos de población, tales como la deriva génica y la consecuente pérdida de diversidad genética. Estás poblaciones, generalmente aisladas del continente y consecuentemente diferenciadas, son tradicionalmente consideradas y gestionadas como unidades independientes de conservación. Sin embargo, en el caso de especies que cuentan con una considerable capacidad dispersiva, dichas poblaciones pueden recibir ocasionalmente inmigrantes provenientes del continente. Esta inmigración, aunque ocasional, jugaría un papel esencial al amortiguar los efectos de la deriva génica y disminuir de ese modo los riesgos asociados a la endogamia, así como al aumentar su potencial evolutivo (Westemeier *et al.* 1998, Madsen *et al.* 1999; Frankham, 2002; Marr *et al.* 2002; Vilà *et al.* 2003; Hogg *et al.* 2006). El objetivo principal de este capítulo de la tesis es testar si las poblaciones oceánicas pueden estar conectadas a sus equivalentes continentales y si por lo tanto, es importante conservar ese flujo para garantizar la futura viabilidad de esas poblaciones reducidas y genéticamente empobrecidas.

En el **capítulo 3** se incorporan el estudio de genes funcionales para, de nuevo, establecer los niveles de diversidad y diferenciación genética. Las medidas moleculares de variación genética neutral, aunque muy útiles para testar diferenciación y flujo genético, proveen una indicación bastante vaga del potencial evolutivo de las poblaciones (Balloux *et al.* 2004; Slate *et al.* 2004; Hansson & Westerberg 2008; Chapman *et al.* 2009). Es el estudio de los genes funcionales el que nos puede aportar una información más precisa acerca de la distribución de la variación genética adaptativa y de los posibles efectos negativos relacionados con la pérdida de diversidad genética (Hedrick 2000). Por otro lado, el estudio comparativo de la distribución de la variabilidad genética entre genes neutrales y funcionales, así como entre poblaciones sujetas a diferentes presiones selectivas, puede ayudarnos a esclarecer cuál es el papel de cada una de las distintas fuerzas evolutivas a la hora de dibujar el perfil genético de las poblaciones. Este es uno de los principales objetivos de la ecología molecular y la biología evolutiva. El problema estriba en que aún hoy, muy pocos genes funcionales han sido caracterizados en las poblaciones naturales. En ese sentido los Genes del Complejo Mayor de Histocompatibilidad (MHC) son una excepción y se convierten en candidatos perfectos para este tipo de estudios. Por un lado, se encuentran entre los genes funcionales mejor estudiados en el mundo animal. Por otro lado, constituyen un componente esencial del sistema inmunológico de los individuos, por lo que están directamente relacionados con la salud individual y la supervivencia (Oliver *et al.* 2009, Radwan *et al.* 2009, Spurgin & Richardson 2010). El objetivo principal de este capítulo es, mediante el estudio de la variabilidad adaptativa (los genes de la clase II β del MHC), analizar las diferencias genéticas funcionales (cuantitativa y cualitativamente) entre las poblaciones insulares (reducidas y empobrecidas genéticamente) y sus equivalentes continentales. A partir de esta información se pretende, por un lado, discutir las consecuencias de tales

diferencias en términos de conservación y por otro lado, describir cuáles han sido las fuerzas selectivas que han dibujado el presente pattrón.

El **cuarto capítulo** de esta tesis tiene como objetivo principal analizar y datar el proceso de colonización y diferenciación de una población insular de un vertebrado de larga vida (el alimoche canario), respecto a su población fuente. El empleo de las herramientas moleculares y las recientes técnicas de análisis nos permiten inferir eventos demográficos acontecidos en el pasado. En este capítulo y a partir del análisis de la diversidad genética neutral mediante técnicas Bayesianas de inferencia demográfica, se esclarece cómo y cuándo pudo originarse una población insular diferenciada y se discute el papel de la actividad humana en dicho origen.

En el último **capítulo (5)**, se da un salto en la escala de estudio y se pasa del análisis poblacional al estudio a nivel individual. Una vez que se han esclarecido las cuestiones que se abordan en esta tesis en los capítulos anteriores (cuáles son las unidades de gestión, cuáles son los niveles de diversidad genética neutral y funcional, si existe un problema genético a nivel poblacional, si hay flujo entre las poblaciones y por lo tanto se pueden potencialmente aliviar dichos problemas), el siguiente paso es examinar las posibles consecuencias de la pérdida de diversidad en las poblaciones, es decir, si existe depresión por endogamia y cuáles son sus efectos. Para ello es necesario un conocimiento profundo de dichas poblaciones, tanto a nivel ecológico como genético. Desgraciadamente, la mayoría de los estudios no alcanzan este nivel de análisis debido a la falta de información individual, especialmente en el caso de especies de larga vida. Una de las aportaciones más importantes de esta tesis es precisamente la determinación de los efectos de la endogamia en la salud individual de una población amenazada e insular, de

una especie de vertebrado de larga vida. Por otro lado, la mayoría de los estudios existentes analizan la depresión por endogamia basándose en medidas indirectas a partir de la variabilidad en marcadores neutrales (como por ejemplo la heterozigosidad individual). Sin embargo, se ha criticado y aún hoy está en debate, la adecuación del empleo de estas medidas indirectas debido a que no se sabe hasta que punto la heterozigosidad medida en varios loci neutrales representa la heterozigosidad genómica de un individuo (Balloux *et al.* 2004; Slate *et al.* 2004; Hansson & Westerberg 2008; Väli *et al* 2008; Chapman *et al.* 2009). Por este motivo es muy recomendable analizar de forma paralela, genes con significado evolutivo. Sin embargo, pocos genes funcionales han sido caracterizados en las especies salvajes y están disponibles para su uso en las poblaciones naturales. En ese sentido y como ya se ha explicado para el capítulo 3, los genes del MHC son la excepción. Su empleo para el estudio de la depresión por endogamia tiene además mucho sentido ya que están directamente relacionados con la capacidad inmune de los individuos, y por lo tanto con su salud y su supervivencia (Oliver *et al.* 2009, Radwan *et al.* 2009, Spurgin & Richardson 2010). La incorporación del estudio de estos genes funcionales y con sentido evolutivo, hace de esta tesis una aportación importante en el campo de la genética y la biología de la conservación de los vertebrados.

Background and Main Questions

A very important proportion of the populations of long-lived vertebrate species on earth are suffering drastic declines and loss of genetic diversity and adaptive potential. This fact can compromise their capability to respond to environmental changes (or even to the implemented measures for their conservation) and consequently accelerate their probability of extinction. One of the main challenges of conservation genetics is to determine the levels of genetic diversity and the potential effects of its loss on the viability of threatened populations. Technological development has encouraged and enabled the use of variable neutral molecular markers in a growing number of wild populations. This has partially allowed addressing these questions. However and despite their indisputable utility, the use of neutral markers entails substantial limitations. Their analysis does not allow evaluating the adaptive genetic variation and, similarly, their use does not permit analyzing the interaction of genetic and environmental effects on the viability of populations. Therefore, there are important gaps in the field of conservation genetics that require the analysis of functional genetics, quantitative genetics and genomics in wild populations (Ouborg *et al.* 2009, 2010; Ouborg 2010).

The present Doctoral Thesis wants to take a step into that direction. The main goal of this study is to provide information about the importance of genetics in the preservation and survival of an insular and highly threatened population of a long-lived vertebrate; the Canarian Egyptian vulture, locally named "*guirre*". Through the analysis of this model species, this study intends to help to understand the role that genetic diversity (typically diminished in Islands) plays on certain demographic parameters (such as the characteristically low insular productivity), and more generally, on the high vulnerability of island populations.

To this end, in addition to the use of molecular techniques, it is necessary to have available ecological information at individual level, which in the case of long-lived vertebrates requires of long-term monitoring. The present study counts on ten years of exhaustive monitoring of the Canarian population of Egyptian vulture, which have provided individual ecological data (morphology, age of recruitment, productivity, survival, among others) of more than 85% of the extant insular population. In addition, more than 20 years of monitoring of some Iberian and the Balearic populations provides samples from other continental and insular areas of the species distribution range, and with ecological and demographic information. These data allow addressing the goals of the present study from a comparative approach.

Hence, this study arose from an already existing research programme with more than ten years, whose main objective is to understand the parameters that are determining the decline and extinction of this unique sub-species, in order to be able to elaborate appropriate measures for its conservation. The first part of this project was materialized in the thesis of Laura Gangoso (2006). Therein, the author delves into the ecology of this population characterized by their condition of insularity. From

this extensive work, some of the processes that are currently accelerating the extinction of the *'guirre'* could be identified. They are mainly associated to the very high non-natural mortality which is directly or indirectly related to human action. In addition, it was observed that the insular nature of the population seems to make it more susceptible to these processes, provoking abnormally low birth rates and a weakened immune response (Donázar *et al.* 2002; Gangoso *et al.* 2009). The next step was to know whether the genetic parameters characteristic of islands (low variability, genetic drift or even inbreeding) were also boosting that vulnerability or affecting the viability of the population.

With the present PhD Thesis it has been able to respond to these questions to a large extent. On the one hand, it is one of the first studies that has genetically characterized, using neutral markers such as microsatellites, a virtually complete population of a long-lived vertebrate. It is one of the few existing works in which the study of the loss of neutral diversity is combined with a parallel analysis of functional genes, and thus potentially adaptive, using the genetic diversity of the Major Histocompatibility Complex responsible for the immune system in vertebrates (MHC). In fact, it is the first study that analyzes this diversity in several natural populations of a highly threatened bird of prey, including the 85% and two generations of individuals of an island population. Genotyping an almost complete population and the study of the MHC allele segregation from parents to offspring, has enabled to partially overcome one of the main problems that scientists confront when studying genes of the MHC in wild populations, especially birds: the simultaneous amplification of multiple copies of the same gene. This problem makes it difficult an accurate calculation of allele frequencies and therefore their analysis. In the present case, the availability of the partial pedigrees of the Canarian Egyptian

vultures allowed to describe linkage groups of alleles from which we could infer the allele frequencies of both the Canarian and the rest of the studied populations. Finally, this thesis is one of the first works where the study of neutral and functional genetics is combined with very precise individual ecological information. As a result, this thesis not only responds to the questions posed in relation to its object of study, but it also brings new and valid conclusions within the field of conservation genetics of vertebrates.

Main contributions

The present results indicate that island populations of highly mobile species can act as metapoblaciones (Haila 1990; Hanski 1999). Despite their isolation and differentiation, island populations can occasionally receive continental individuals (**Chapter 2**). This immigration, although sporadic, has not prevented however, the phenotypic and genetic differentiation of the Canarian Egyptian vultures (described as a distinct subspecies (*N. p. majorensis*, Donázar *et al.* 2002)) (**Chapter 4**). On the contrary, this gene flow can have beneficial effects in this, and in the insular populations in general, by increasing levels of genetic diversity and evolutionary potential and reducing the risks associated with genetic drift (Westemeier *et al.* 1998, Madsen *et al.* 1999; Frankham, 2002; Keller *et al.* 2002; Vilà *et al.* 2003; Hogg *et al.* 2006; Ortego *et al.* 2008). The existence of immigration, although rare, has important implications in terms of conservation. First, it involves that the conservation of island populations of vertebrates species with dispersive capacity, may depend on the state of conservation of their continental counterparts, even when there are clear differentiation processes. On the other hand, it raises important

practical issues. Isolated and diversified island populations have been traditionally regarded as separate management units, however the results thrown in this thesis indicate that, on certain occasions as in the case of highly mobile species, taking for granted that independence and ignoring the connection to the continent, can limit the ecological vision of the problem and therefore prevent from the elaboration of a more adequate and successful management plan.

Preserve or promote the entry of 'new genes' in the island populations can partly alleviate the negative effects of the loss of genetic diversity. All island populations have suffered a bottleneck during their foundation what determines a more or less deep genetic impoverishment, depending on the number of founders (Pimm *et al.* 1988, Frankham 1995). Reduced sizes of populations may in turn, facilitate loss by drift. In this way, islands are naturally more likely to suffer the effects of inbreeding than their continental counterparts (Frankham 1997, 1998). The population declines that most insular demes are subjected at present, due to human activity, could also be increasing this trend. In the present study, it is provided empirical evidence that show how these reduced and genetically impoverished entities are subject to the effects of inbreeding, and become more vulnerable to extinction (**Chapter 5**). On the one hand, it is observed that loss of diversity affects both neutral loci and genes under selection. The results indicate that drift dominates as evolutionary force above forces of adaptive selection (**Chapter 3**). Island demes lose adaptive potential and consequently are unable to respond to environmental changes such as, for example, the arrival of new pathogens. Previous results relating an increase in abundance of viruses and bacteria in the islands and the mainland, warn about the exponential growth of pathogens that certain animal populations are suffering, particularly those belonging to the scavengers species (Blanco *et al.* 2007, Lemus & Blanco 2009a,

Lemus *et al.* 2008; Gangoso *et al.* 2009). This increase may have very different consequences in the two types of populations, becoming catastrophic on islands where individuals lack of the required genetic diversity to respond to these new threats. The present study evidences how a genetically impoverished population may be unable to respond to an environmental change favoured by human action (**Chapter 3**).

Apart from reducing the evolutionary potential, the loss of genetic diversity may directly affect the parameters that determine the individual fitness, and finally the viability of populations. There is an increasingly number of studies suggesting the existence of negative effects caused by the loss of genetic diversity in natural populations (generally called inbreeding depression) (see the review of Chapman *et al.* 2009). However, it is still unknown how these mechanisms operate since there are to date, very few studies that describe cause-effect relationships between genetic diversity and the ecological parameters related to fitness (e.g. Hansson *et al.* 2001; Acevedo-Whitehouse *et al.* 2006; Ortego *et al.* 2007; Luikart *et al.* 2008; Blomqvist *et al.* 2010). In addition, most of the studies carried out thus far have used indirect measures of inbreeding, particularly measures of individual diversity based on few neutral markers. These values can poorly represent the levels of genomic diversity (Balloux *et al.* 2004; Slate *et al.* 2004; Hansson & Westerberg 2008; Chapman *et al.* 2009). The main advantage that the present study has with respect to most of the equivalent articles previously published are: 1) it is employed a relatively large set of neutral markers (22 loci) (**Chapter 1**), whose heterozygosity values are significantly correlated, suggesting that they can be used as good indicators of the overall genetic variability (Szulkin *et al.* 2010), 2) it is measured diversity at neutral loci and also, as already mentioned above, it is estimated the effects of loss of functional diversity on the individual fitness, 3) both measures of genetic

diversity (neutral and functional) are correlated as well, what may be another indicative of the validity of our neutral markers as indirect indicators of the genomic variability, 4) it is known the degree of kinship between the breeding pairs, what provides another and more direct measure of inbreeding, and 5) the study has been carried out on a sample rather representative of the population (85%). All these elements give consistency to the present results, which suggest that there is a significant relationship between genetic diversity and the individual fitness of the Canarian Egyptian vultures (**Chapter 5**). First, it is noted that the diversity at genes linked to the immune system is significantly related to the productivity of individuals, which corroborates that the immune response is one of the most important characteristics determining the individual fitness (**Chapter 3**). Second, it is observed that there is a correlation between inbreeding (indirectly measured through the individual neutral heterozigosidad) and the age of recruitment. Hence, less 'endogamous' (or more heterozygotes) individuals recruit earlier. The delay in the age of recruitment in long-lived species can accelerate their risk of extinction (Weimerskirch 1992; Congdon *et al.* 1993; Saether & Bakke 2000; Eberhardt 2002; Grande *et al.* 2009). These results could partly explain the high risk of extinction typically observed in island populations of long-lived bird species.

Globally, the present data demonstrates that the genetic deterioration in small populations has a negative impact on the individual fitness and the population viability, thus increasing the population's risk of extinction (e.g. Keller 2002; Brook *et al.* 2002; Spielman *et al.* 2004; Frankham 2005; Blomqvist *et al.* 2010). These results represent an empirical evidence of how the conjunction of deterministic factors, generally related to human activity (non-natural mortality caused by direct or indirect persecution or introduction of pathogens) and the stochastic genetic factors (loss of adaptive potential and inbreeding depression) can irremediably

multiply the probability of extinction of a population, if not appropriate measures are taken in time.

In the present case, there is no doubt that human activity is accelerating the decline of the canarian Egyptian vulture population. However, human action was also responsible for its colonization and later differentiation (**Chapter 4**). Such colonization is very recent (only about 2500 years), which make us to wonder if we may be witnessing a natural event of colonization-extinction. This question leads, in turn, to a more general matter: to what extent are large vertebrates able to survive on the islands? The arrival of a predictable and abundant food source allowed the founding of the Egyptian vulture in the Canary Islands. Similarly, the decline or demise of this source of energy due to changes in the island economies, could largely determine the extirpation of the species from most of the islands of the archipelago, because the species was unable to respond to those changes. The occasional arrival of continental individuals, potential re-colonizers, indicates that the present process of extinction could be reverted if favourable environmental conditions in the islands return. Of course, for this to happen, it would be necessary to maintain healthy and large continental populations (since the arrival of these individuals must be a very occasional event) and nowadays these populations are also in serious decline. This thesis has allowed drawing the entire episode from the foundation to the near-extinction of an insular population. The observations suggest that these entities can be dynamic, ephemeral, and very vulnerable.

The question that I guess arises in the mind of the reader is: what should we do to preserve the *'guirre'*? This thesis is not intended to be a management manual, but it can and must bring up questions of practical utility. Nevertheless, the answer to that question is complex and we would need more than the few lines

of this text to answer it. I would like, though, open some interrogates about the applied management dealing with cases like the one here. Obviously, the first thing we need to address for the conservation of any population is the removal of the direct causes of its decline, which can be achieved with relatively simple measures (see Laura Gangoso' thesis for more detail). But, focusing on the purely conservation genetics: how do we reduce the risks associated with the loss of diversity in a genetically impoverished population? From what perspective, we must think about carrying out measures such as translocations or reintroductions? Answering these questions requires a very thorough understanding, at both the genetic and ecological level, of the threatened populations. There are no magic formulas and each population should be studied independently since there are many variables that come into play and will be different in each case. Projections using demographic models are very useful, because they permit to predict the moment when the application of such measures would be recommended, to significantly improve the population's viability (see for example McCullough *et al.* 1996 or Dobson *et al.* 1992). The fact is that there barely are examples in which immigration (or other type of restoration of the gene flow between populations) is applied to relieve genetic problems, with the exception of the few cases already mentioned (see general introduction). The question that I would like to leave as the culmination of this synthesis is: is it now time to start to more widely apply such measures, in conjunction with other protective measures, on reduced and threatened populations before they reach critical levels of inbreeding?.

Next steps

The results obtained in this Thesis open new matters that would be interesting to deal with in the near future. Based on the observed effects of genetic diversity on the age of recruitment, a model aiming to predict the demographic consequences of such effect on island populations in a long term, could be developed. Such a study could quantify the role of inbreeding in the extinction risk of an insular population. There barely are such sorts of studies so far, but their development is one of the main priorities within the conservation genetics area, summarized by Frankham (2010a).

Our data would allow us to analyse whether, apart from the observed effects, the loss of genetic diversity is affecting the offspring survival. This analysis has not been included in this Thesis, but it would be highly recommended since it would complement the existing results. In addition, it would be interesting to include other items, related to the insular syndrome and not previously addressed, that could be affecting the individual fitness. For example, it would be relevant to verify whether the individual size, characteristically larger on islands, is correlated with the individual fitness. A preliminary analysis has revealed some significant correlation. This correlation is very interesting because it would explain the trend towards gigantism observed in the Canarian Egyptian vultures and also characteristic of many insular taxa (Carlquist 1972; Lomolino 2005). This relationship would also clarify how the observed differentiation (increased size in the *'guirres'*) have been able to occur so quickly (less than 200 generations, **Chapter 4**) which is hardly understandable by drift alone. It would also empirically

demonstrate, for the first time, one of the theories that explains the general trend to gigantism in many insular species: reduction of inter-specific competition and increasing of intra-specific competition (Grant 1965; Carlquist 1972; Lomolino 2005).

Another line of study that has recently been recommended to be implemented within the field of conservation genetics is the quantitative genetics. The adaptive capacity of species is more related to quantitative genetics than to molecular variation; however, there are very few estimates of its variability in natural populations of endangered species (Frankham 2010b). The deep biological knowledge of the Egyptian vulture, the availability of individual morphological and ecological data in virtually the whole population and from at least two generations, make the Canarian population to be an ideal model for carrying out this type of study, inexistent to date in wild populations of long-lived species.

Antecedentes y preguntas

Una proporción muy importante de las poblaciones de vertebrados de larga vida existentes en el planeta están sufriendo un proceso drástico de declive y pérdida de diversidad genética y potencial adaptativo. Este hecho puede llegar a comprometer su capacidad de respuesta ante los cambios ambientales (e incluso ante las medidas aplicadas para su conservación) y consecuentemente, acelerar su probabilidad de extinción. Uno de los principales retos de la genética de la conservación es determinar los niveles de diversidad genética y los potenciales efectos de su pérdida, sobre la viabilidad de las poblaciones amenazadas. El desarrollo tecnológico ha habilitado y estimulado el uso de marcadores genéticos neutrales variables en un número cada vez mayor de poblaciones, lo que ha permitido responder en parte a esas preguntas. Sin embargo y a pesar de su ineludible utilidad, el uso de estos marcadores entraña substanciales limitaciones ya que no permite evaluar la variación genética adaptativa y del mismo modo, tampoco permite analizar la interacción de los efectos genéticos y ambientales sobre la viabilidad de las poblaciones. Quedan por lo tanto importantes lagunas que cubrir en el campo de la genética de la conservación que requieren del análisis de la genética funcional, la genética

cuantitativa y la genómica en las poblaciones salvajes (Ouborg *et al.* 2009, 2010; Ouborg 2010).

La presente tesis quiere dar un paso en esa dirección. El objeto motor de este trabajo es resolver los interrogantes existentes en torno a la importancia de la genética en el estado de conservación y supervivencia de una población insular y altamente amenazada de un vertebrado de larga vida; el alimoche canario o guirre. Mediante el análisis de este modelo de estudio pretendemos ayudar a comprender que papel juega la diversidad genética, típicamente reducida en las islas, sobre determinados parámetros demográficos (tales como la característicamente baja productividad insular), y a nivel más general, sobre la elevada vulnerabilidad propia de las poblaciones isleñas.

Para poder lograr estos objetivos, a parte del empleo de las técnicas moleculares, es necesario disponer de información ecológica a nivel individual, la cuál en el caso de un vertebrado de larga vida requiere de su seguimiento a largo plazo. El presente trabajo cuenta con diez años de exhaustivo monitoreo de la población canaria de alimoche, gracias al cual se dispone de datos ecológicos individuales (morfología, edad de reclutamiento, productividad, supervivencia, entre otros) de más del 85% de la población residente en las islas. Además, los más de 20 años de seguimiento de varias poblaciones Ibéricas y de la Balear permiten disponer de muestras de otras áreas de distribución, continentales e insulares, y de información ecológica individual y demográfica lo cual permite abordar este estudio desde una aproximación comparativa.

Este estudio no partía pues de cero si no que surgía de un proyecto ya vigente y con más de diez años de duración, cuyo objetivo principal es comprender los parámetros que están determinando el declive y la extinción de una subespecie única, para poder establecer las medidas adecuadas para su

conservación. La primera parte de este proyecto se materializó en la tesis de Laura Gangoso (2006). En ella la autora profundiza en la ecología de esta población caracterizada por su condición de insularidad. A partir de este extenso trabajo se pudieron esclarecer algunos de los procesos que están llevando al guirre a la extinción. Éstos están relacionados principalmente con la elevada mortalidad no natural debida, de manera directa o indirecta, a la acción humana. Además, se observó que el carácter insular de la población parece hacerla más susceptible a esos procesos determinando unas tasas de natalidad anormalmente bajas y una debilitada respuesta inmune (Donázar *et al.* 2002; Gangoso *et al.* 2009). El siguiente paso era saber si los parámetros genéticos característicos de las islas (bajos niveles de variabilidad, deriva génica o incluso la endogamia) estaban potenciando también esa vulnerabilidad o afectando a la salud de la población.

Con la presente Tesis Doctoral se ha podido dar respuesta en buena parte a estos interrogantes y representa un avance cuantitativo en la dirección indicada. Por un lado, es uno de los primeros estudios en caracterizar genéticamente (usando marcadores neutrales microsatélites) una población prácticamente completa, de un vertebrado de larga vida. Es uno de los pocos trabajos existentes en el que el estudio de la pérdida de diversidad neutral se combina con el análisis paralelo de genes funcionales y potencialmente adaptativos como son los del Complejo Mayor de Histocompatibilidad (MHC). Es, de hecho, el primer estudio que analiza la diversidad de los genes del MHC en varias poblaciones naturales de una rapaz en peligro, incluyendo el 85% y dos generaciones de individuos de una población insular. El genotipado de una población casi completa y el estudio de la segregación de los alelos de padres a hijos, ha permitido solventar parcialmente uno de los principales problemas al que los científicos nos enfrentamos a la hora de estudiar los genes del MHC en especies salvajes, especialmente aves: la amplificación

simultánea de varias copias del mismo gen. Este problema dificulta el cálculo de las frecuencias alélicas reales y por lo tanto el análisis de las mismas. En el presente caso, la disponibilidad del pedigrí parcial de la población canaria de alimoche permitió describir grupos de ligamiento de alelos entre las copias del gen e inferir las frecuencias alélicas tanto en Canarias como en el resto de las poblaciones. Por último, esta tesis es uno de los primeros trabajos en el que el estudio de la genética neutral y funcional se combina con una información ecológica individual muy precisa. Como resultado, no solo se responden a las preguntas que se planteaban en relación al objeto de estudio de esta tesis, si no que se aportan conclusiones nuevas y válidas dentro del marco de la genética de la conservación de los vertebrados.

Principales aportaciones

Los resultados de esta tesis indican que las poblaciones insulares de especies con capacidad dispersiva pueden actuar como metapoblaciones (Haila 1990; Hanski 1999). A pesar de su aislamiento y diferenciación, las poblaciones insulares pueden recibir individuos continentales de manera ocasional (**capítulo 2**). Esta inmigración, aunque esporádica, no ha impedido empero, la diferenciación fenotípica y genética de los alimoches canarios (descritos como una subespecie diferenciada (*N. p. majorensis*, Donázar *et al.* 2002)) (**capítulo 4**). Por el contrario, este flujo génico puede tener efectos positivos en ésta, y en las poblaciones insulares en general, al aumentar los niveles de diversidad genética y su potencial evolutivo, y reducir los riesgos asociados a la deriva génica (Westemeier *et al.* 1998, Madsen *et al.* 1999; Frankham, 2002; Keller & Waller 2002; Vilà *et al.* 2003; Hogg *et al.* 2006; Ortego *et al.* 2008). La existencia de inmigración, aun cuando

rara, tiene importantes repercusiones en términos de conservación puesto que implica, por un lado, que el estado de conservación de las poblaciones insulares, de especies de vertebrados con capacidad dispersiva, puede depender del estado de conservación de sus equivalentes continentales, aun cuando existan procesos claros de diferenciación. Por otro lado, plantea cuestiones prácticas importantes. Las poblaciones insulares aisladas y diversificadas han sido tradicionalmente consideradas unidades independientes de gestión, sin embargo los resultados arrojados en esta tesis indican que en determinadas ocasiones, como en el caso de especies muy móviles, dar por sentado esa independencia e ignorar la conexión con el continente, puede limitar la visión ecológica del problema y comprometer una gestión adecuada y más exitosa.

Preservar o favorecer la entrada de 'nuevos genes' en las poblaciones insulares puede aliviar en parte los efectos negativos de la pérdida de diversidad genética. Todas las poblaciones insulares sufren un cuello de botella en su fundación lo que determina un empobrecimiento genético más o menos profundo, dependiendo del número de colonizadores (Pimm *et al.* 1988, Frankham 1995). Los tamaños reducidos de estas poblaciones facilitan a su vez la pérdida por deriva. De este modo las islas tienen, de forma natural, más probabilidades de sufrir los efectos de la endogamia que sus equivalentes continentales (Frankham 1997, 1998). Los declives poblacionales a los que la mayoría de las poblaciones insulares se ven sometidas en la actualidad, debido a la actividad humana, no hacen sino incrementar esta tendencia. En el presente trabajo se evidencia como estas entidades, reducidas y genéticamente empobrecidas, son víctimas de los efectos de la endogamia haciéndose más vulnerables a la extinción (**capítulo 5**). Por un lado se observa que la pérdida de diversidad afecta tanto a genes neutrales como a genes sujetos a selección. Los resultados sugieren que la deriva predomina como fuerza evolutiva por

encima de las fuerzas de selección adaptativa. Así, las poblaciones insulares pierden potencial adaptativo y son incapaces de responder a los cambios ambientales como, por ejemplo, la llegada de nuevos patógenos. El incremento que se ha venido observando en la abundancia de virus y bacterias tanto en las islas como en el continente puede tener consecuencias muy distintas en los dos tipos de poblaciones, pudiendo alcanzar niveles catastróficos en las islas donde los individuos carecen de la diversidad necesaria para responder a esas nuevas amenazas (**capítulo 3**). El presente trabajo muestra un ejemplo claro de un cambio ambiental favorecido por la acción humana, al que una población genéticamente empobrecida es incapaz de responder.

A parte de disminuir el potencial adaptativo, la pérdida de diversidad puede afectar de manera directa a los parámetros que determinan la salud y la eficacia biológica de los individuos, y finalmente la viabilidad de las poblaciones. Existen cada vez más estudios que sugieren la existencia de efectos negativos causados por la pérdida de diversidad genética en las poblaciones naturales (conocido en conjunto como depresión por endogamia) (ver la revisión de Chapman *et al.* 2009). Sin embargo, se desconoce aún cómo operan dichos mecanismos ya que hasta la fecha, hay muy pocos trabajos que describan relaciones de causa-efecto entre la diversidad genética y los parámetros ecológicos relacionados con la eficacia biológica (e.g. Hansson *et al.* 2001; Acevedo-Whitehouse *et al.* 2006; Ortego *et al.* 2007; Luikart *et al.* 2008; Blomqvist *et al.* 2010). Además, la mayoría de los estudios llevados a cabo han utilizado medidas indirectas de la endogamia, especialmente medidas individuales de diversidad basadas en unos pocos marcadores neutrales. Estos valores pueden representar muy pobremente los niveles de diversidad genómica (Balloux *et al.* 2004; Slate *et al.* 2004; Hansson & Westerberg 2008; Chapman *et al.* 2009). La ventaja de este estudio respecto a la mayoría de los trabajos equivalentes publicados hasta la fecha estriba en que: 1)

se emplea una batería relativamente amplia de marcadores (22 loci; **capítulo 1**), cuyos valores de heterocigosidad se encuentran además correlacionados significativamente, lo que sugiere que pueden ser buenos indicadores de la variabilidad global (Szulkin *et al.* 2010), 2) no solo se mide la diversidad a nivel neutral si no que, como ya se ha mencionado, se estiman los efectos de la pérdida de diversidad funcional sobre la salud de los individuos, 3) ambas medidas de diversidad (neutral y funcional) se encuentran a su vez correlacionadas, lo que puede ser otro indicativo más de la validez de los marcadores neutrales como indicadores indirectos de la variabilidad genómica, 4) se conoce el grado de parentesco de las parejas reproductoras lo que aporta otra medida más directa de la endogamia y 5) el estudio se ha llevado a cabo en una muestra más que representativa de la población (el 85%). Todos estos elementos dan consistencia a estos resultados, los cuales sugieren que existe una relación significativa entre la diversidad génica y la eficacia y la salud individual de los alimoches canarios (**capítulo 5**). Por un lado, se observa que la diversidad de los genes relacionados con el sistema inmune está significativamente relacionada con la productividad de los individuos, lo que corrobora que la capacidad de respuesta inmune es una de las características más importantes que determina la eficacia individual. Por otro lado se detecta una correlación entre la endogamia (medida indirectamente mediante la heterozigosidad neutral individual) y la edad de reclutamiento, de tal modo que aquellos jóvenes menos 'endogámicos' o más 'heterocigotos' se incorporan antes a la población reproductora. El retraso en la edad de reclutamiento en especies de larga vida, puede acelerar su riesgo de extinción (Weimerskirch 1992; Congdon *et al.* 1993; Saether & Bakke 2000; Eberhardt 2002; Grande *et al.* 2009). Estos resultados podrían explicar en parte los elevados riesgos de extinción típicamente observados en las poblaciones insulares de aves de larga vida.

Globalmente, los datos obtenidos demuestran que el deterioro genético en las poblaciones reducidas tiene un efecto negativo en la salud individual y poblacional, aumentando así su riesgo de extinción (e.g. Keller & Waller 2002; Brook *et al.* 2002; Spielman *et al.* 2004; Frankham 2005; Blomqvist *et al.* 2010). Estos resultados representan una evidencia empírica de cómo la conjunción de los factores determinísticos, generalmente relacionados con la actividad humana (mortalidad no natural por persecución directa o indirecta o introducción de patógenos) y de los factores estocásticos de origen genético (pérdida de potencial evolutivo y depresión por endogamia) puede multiplicar irremediablemente la probabilidad de extinción de una población, si no se toman las medidas adecuadas a tiempo.

En el caso del guirre, es indiscutible que la actividad humana ha acelerado el declive de la población, sin embargo, fue también responsable indirecto de su colonización y posterior diferenciación (**capítulo 4**). Esta colonización es muy reciente (apenas 2500 años), lo cual nos plantea si no estaremos siendo testigos de un evento natural de colonización-extinción, lo que a su vez nos conduce a una cuestión más general: ¿hasta que punto pueden los grandes vertebrados sobrevivir en las islas? La llegada de una fuente de alimento abundante y predecible permitió el asentamiento del alimoche en las islas Canarias. Del mismo modo, la disminución o desaparición de dicha fuente energética debido a los cambios en las economías insulares, ha podido determinar, en gran parte, la extirpación de la especie de la mayoría de las islas del archipiélago, la cual ha sido incapaz de responder a los cambios acontecidos. La llegada ocasional de individuos continentales, potenciales recolonizadores, indica que el presente proceso de extinción podría revertirse si las condiciones ambientales en las islas volvieran a ser favorables. Claro que para ello sería necesario el mantenimiento de poblaciones continentales sanas y de gran tamaño (puesto que la llegada de estos individuos

debe de ser un evento muy ocasional) y en la actualidad estas poblaciones están también en serio declive. La presente tesis ha permitido dibujar el escenario completo desde el origen hasta la casi-extinción de una población insular y sugiere que estas entidades pueden ser dinámicas, efímeras y muy vulnerables.

La pregunta que imagino surge a continuación en la mente del lector es: ¿qué debemos hacer para preservar el guirre? Esta tesis no pretende ser un manual de gestión, pero si puede y debe plantear cuestiones de utilidad práctica. No obstante, la respuesta a esa pregunta es compleja y nos llevaría más de las escasas líneas de este texto para contestarla. Sí me gustaría, empero, abrir algunos interrogantes acerca de la gestión aplicada de casos como el que nos ocupa. Es obvio, que lo primero que debemos abordar para la conservación de cualquier población, es la eliminación de las causas directas del declive, lo cual puede conseguirse con medidas relativamente sencillas (véase la tesis de Laura Gangoso para más detalle). Pero, ya centrándonos en la genética de la conservación: ¿como reducimos los riesgos asociados a la pérdida de diversidad en una población genéticamente empobrecida?, ¿a partir de qué momento debemos plantearnos llevar a cabo translocaciones o reintroducciones? La respuesta a estas preguntas requiere de un conocimiento muy exhaustivo tanto a nivel genético como ecológico de las poblaciones, no hay fórmulas mágicas y cada población ha de ser estudiada independientemente puesto que son muchas las variables que entran en juego y serán distintas en cada caso. La elaboración de proyecciones mediante modelos demográficos es de gran utilidad, pues permiten predecir a partir de que momento serían recomendables este tipo de medidas y como pueden mejorar la viabilidad de las poblaciones (ver por ejemplo McCullough *et al.* 1996 o Dobson *et al.* 1992). La realidad actual es que, exceptuando unos pocos casos ya mencionados (ver introducción general), apenas existen ejemplos en los que la inmigración (u otro tipo de

reestablecimiento del flujo génico entre poblaciones) se haya aplicado para aliviar problemas genéticos. La cuestión que me gustaría dejar como colofón de la síntesis de este trabajo, es, si no ha llegado el momento de empezar a aplicar más ampliamente este tipo de medidas, en conjunción con otras medidas protectoras, en las poblaciones reducidas y amenazadas, antes de que alcancen niveles críticos de endogamia.

Los siguientes pasos

Los resultados obtenidos en esta tesis plantean nuevas cuestiones que sería interesante abordar en el futuro próximo. A partir de los datos del efecto de la diversidad genética sobre la edad de reclutamiento, se podría elaborar un modelo que nos permitiera predecir las consecuencias demográficas a largo plazo de dicho efecto en poblaciones insulares como la que nos ocupa. Este tipo de estudio permitiría cuantificar el papel de la endogamia en el riesgo de extinción de una población insular. Apenas existen trabajos de esta índole pero su elaboración es una de las principales prioridades dentro del área de la genética de la conservación resumida por Frankham (2010a).

Nuestros datos permitirían analizar si, además de los efectos observados, la pérdida de diversidad genética está afectando a la supervivencia de la descendencia de cada individuo. Ese análisis no ha sido incluido en esta tesis pero su estudio sería muy recomendable ya que completaría los resultados obtenidos. Además, sería interesante incluir otros elementos relacionados con la insularidad que pudieran estar afectando a la salud y a la eficacia de los individuos y que no se han abordado hasta la fecha. Por ejemplo, sería relevante comprobar si el tamaño

característicamente mayor de los individuos insulares, se encuentra correlacionado con el éxito o la calidad individual. Un análisis preliminar nos ha revelado cierta correlación, lo cual es muy interesante porque explicaría la tendencia al gigantismo, observada en los alimoches canarios, y característica de muchos taxones insulares (Carlquist 1972; Lomolino 2005). Esa relación aclararía también que esa diferenciación (aumento del tamaño de los guirres) se haya podido producir de un modo tan rápido (menos de 200 generaciones, **capítulo 4**) lo cual es muy difícilmente explicable solo por deriva. Además, demostraría de forma empírica, por primera vez, una de las teorías con la que la biogeografía de islas explica la tendencia general al gigantismo observada en muchas especies insulares: la reducción de la competencia inter-específica y el aumento de la competencia intra-específica (Grant 1965; Carlquist 1972; Lomolino 2005).

Otra de las líneas de trabajo que es recomendable empezar a aplicar con mayor frecuencia dentro del campo de la genética de la conservación, es la genética cuantitativa. La capacidad evolutiva de las especies está más relacionada con la genética cuantitativa que con la variación molecular, sin embargo, apenas existen estimas de su variabilidad en las poblaciones naturales de especies amenazadas (Frankham 2010b). El profundo conocimiento biológico de la población canaria de alimoche, la disponibilidad de datos morfológicos y ecológicos individuales en un porcentaje muy representativo de la población y en al menos dos generaciones, la convierten en un modelo ideal para llevar a cabo este tipo de estudios, inexistentes hasta el momento en poblaciones salvajes de especies de larga vida.

An adult Canarian Egyptian vulture (Neophron percnopterus majorensis) poses on a volcanic rock in Fuerteventura, Canary Islands

Guirre adulto posado sobre una roca volcánica en Fuerteventura, Islas Canarias

naranja es la arcilla que tiñe sus plumas
naranja los líquenes que adornan la lava fría
naranja intenso el sol que se oculta tras un mar en calma
pintando de naranja también, la montaña sagrada Tindaya

CHAPTER ONE

Isolation and Characterization of 18 Microsatellite Loci in the Egyptian vulture (*Neophron percnopterus*)

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Running title: Microsatellites from the Egyptian vulture

Aislamiento y caracterización de 18 microsatellites en el Alimoche (*Neophron percnopterus*)

Resumen

El uso de marcadores específicos es muy recomendable, especialmente cuando se trata de obtener estimas de variabilidad genética en poblaciones amenazadas. Los marcadores heteroespecíficos suelen presentar disminuidos niveles de polimorfismo cuando se aplican a otras especies distintas de la que se ha empleado para su diseño, lo que se denomina como 'ascertainment bias'. Por otro lado, en la última parte de esta tesis analizamos el parentesco y grado de endogamia en la población Canaria de Alimoche, lo que implicaba disponer de un gran número de marcadores polimórficos. Antes de comenzar nuestro estudio contábamos con 14 microsatélites previamente diseñados en el Quebrantahuesos (*Gypaetus barbatus*). De todos ellos tan sólo 5 amplificaron correctamente y fueron polimórficos y pudieron, por lo tanto, ser empleados con éxito en nuestro estudio. Además de esos 5 marcadores, desarrollamos dos librerías completas de microsatélites específicos para el Alimoche, a partir de las cuales obtuvimos un total de 18 loci polimórficos, tras descartar aquellos en desequilibrio de ligamiento o con alelos nulos.

En este capítulo se describen los protocolos seguidos para la creación de las librerías enriquecidas y la amplificación de los fragmentos. Además, se resumen los niveles observados de heterozigosidad y riqueza alélica en la subespecie Canaria (*N. p. majorensis*) y en la Europea (*N. p. percnopterus*) (0.46 y 3.9, 0.51 y 4.7 respectivamente).

Abstract

We developed 18 new microsatellite loci for the endangered Egyptian vulture (*Neophron percnopterus*). Microsatellite loci were screened for variation in two different populations belonging to separate subspecies: the nominal *N. p. percnopterus* and the Canarian *N. p. majorensis*. Mean expected heterosygosities were respectively 0.51 and 0.46, while the mean number of alleles per locus was 4.7 and 3.9. These new markers allow further genetic studies for the endangered Canarian Egyptian Vulture.

Introduction

Large avian scavengers are sharply decreasing all around the Old World, mainly due to human direct persecution and accidental poisoning and electrocution in power lines (Koenig 2006). Egyptian vultures (*Neophron percnopterus*) were continuously distributed from the Iberian Peninsula to India and from the Magreb to South Africa. At the present, the species has disappeared in most developed countries in southern Europe, Middle East and North Africa, where only small isolated populations persist (BirdLife International 2007). In addition, it vanished from many islands such as Cyprus, Crete, and Malta in the Mediterranean (Levy 1996) and in most of the Canarian and Cape Verde islands in the Macaronesia (Donázar *et al.* 2005). The relict Canarian population is currently considered as a differentiated subspecies (*N. p. majorensis*), and it is heavily threatened by human-induced mortality. Due to its small size, it may also face the risks of inbreeding (Donázar *et al.* 2002a, Kretzman *et al.* 2003). Here, we describe the isolation and characterisation of 18 microsatellites loci for conservation genetic analyses of the species. The development of species-specific DNA markers will allow the genetic characterisation of the surviving populations, the estimation of the possible levels of inbreeding, the genealogical relationships between individuals and the degree of gene flow between populations.

Methods and Results

We constructed an enriched genomic library as described by Glenn *et al.* (2000). DNA extractions were performed from blood samples and approximately 10 μg of high molecular weight DNA was isolated by phenol-chloroform extraction (Sambrook *et al.* 1989). Simultaneous restriction-ligation of genomic DNA was carried out using the RsaI restriction enzyme and double stranded

linker-adapted primers according to Hamilton *et al.* (1999). Ligated DNA was enriched with a biotin-labelled probe mixture consisting of $(GT)_{10}$ and $(CT)_{10}$ at 10 µM each. DNA fragments with repetitive sequences were then selectively captured by streptavidin-coated Dynabeads (Oxoid) and separated by a magnetic field. Enriched DNA was eluted in 200 µl dH_2O from the magnetic beads and concentrated by vacuum centrifugation to a final concentration of ~100ng/µl. DNA was then reamplified by polymerase chain reaction (PCR), purified and ligated into a cloning vector using pGEM-T Easy Vector II (Promega). A total of 750 positive clones were screened and checked for inserts using ABI PRISM BigDye Terminator Cycle kit (Applied Biosystems) and resolved on an ABI 3100 Genetic Analyser (Applied Biosystems). Primer pairs for 88 potentially usable microsatellite loci were designed using the software package Primer3. Polymorphism was tested by multiplex PCR reactions performed in 20 µl total volume, which include 50 ng of DNA, 2 mM of $MgCl_2$, 0.25 µM of each primer, 200 µM dNTP's, 1x reaction buffer [75 mM Tris-Hcl, 20 mM $(NH_4)_2SO_4$] and 0.5 units Taq polymerase (BIOTAQ). Reaction conditions were as follows: an initial denaturation step of 5 min at 95°C, 6 cycles consisting of 45s at 92°C, 45 s from 48 to 56°C depending on each microsatellite, and 45s at 72°C followed by an additional 22 cycles consisting of 30s at 92°C, 30s from 52 to58°C according the marker and 30s at 72°C (Table 1). Microsatellite variability was assessed in 30 individuals from Fuerteventura Island (Canary Island) and 30 from the Navarra population (north of Spain). Individuals were genotyped by assessing allele size on an ABI 3100 Genetic Analyser (Applied Biosystems) using forward primers labelled with FAM (Sigma) and NED, PET and VIC (Applied Biosystems). Allele scoring was carried out using the GENEMAPPER software version 3.5 (Applied Biosystems).

The program MICROCHECKER version 2.2.3 (van Oosterhout *et al.* 2004) was used to identify possible null alleles, large allele

dropout, scoring error due to stutter peaks, and possible typographic errors. Deviations from Hardy-Weinberg equilibrium, heterozygote deficits and linkage disequilibrium were tested using the program GENEPOP 3.4 (updated version 1.2 described in Raymond and Rousset 1995).

The presence of null alleles was detected for two loci (Np166 and Np244). These two loci showed deviation from Hardy Weinberg Equilibrium after correction for multiple tests in the Canarian sample, but only the Np166 in the Spanish sample, together with the Np238. A single pair of loci was significantly linked in both samples after correction for multiple comparisons (Np302 and Np39 $p<0.00001$). Thus, in the final analysis, the less polymorphic marker (Np302, Acc.No: EU195846) was excluded. Three of the 22 amplified microsatellites were monomorphic in both populations (Np122, Acc.No: EU195849), Np175 (Acc.No: EU195857) and Np240 (Acc.No: EU195853) and thus, also excluded.

The mean numbers of alleles per locus for the two sampled populations were 4.7 and 3.9 and the mean expected heterosygosities were 0.51 and 0.46 (Spanish and Canarian respectively) (Table 2). The apparently lower values of heterozygosity and number of alleles found in the Canarian Island suggest that this population may suffer inbreeding depression as previously proposed (Kretzman *et al.* 2003).

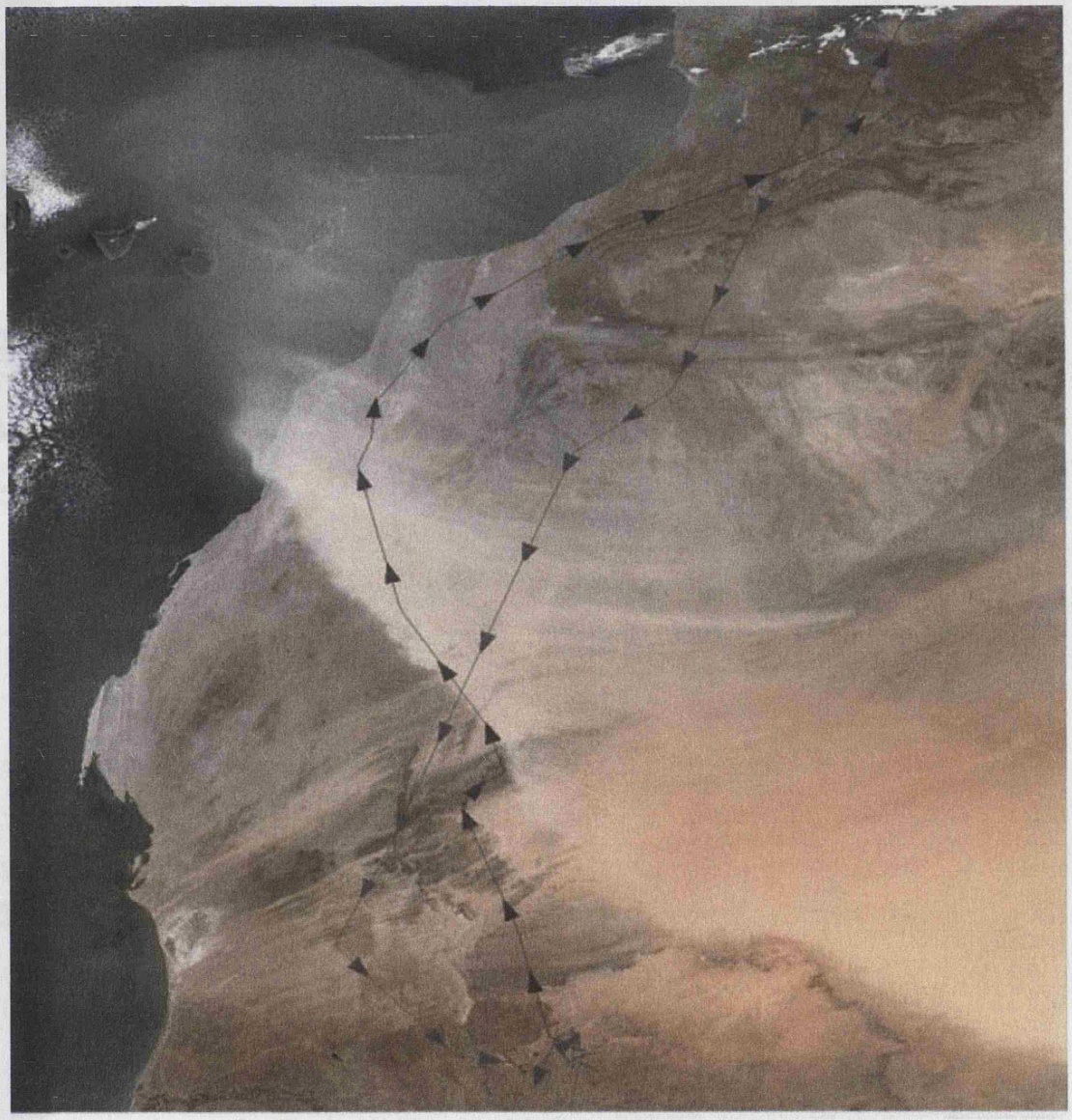
Table 1 Details of the 18 microsatellite markers developed in the study. GenBank Accession number, 5'→3' Forward and Reverse primer sequence, MP numbers indicate loci sharing multiplex PCR reactions, touch-up annealing temperature (T^a), final $MgCl_2$ concentration (Mg) and number of cycles.

Locus	Repeat motif	Acc.No	Left primer sequence (5'-3')	Rigth primer sequence (5'-3')	MP	T_a (°C)	Cycles	Mg (mM)
Np38	$(GT)_{11}GG(GA)_6$	EU195837	VIC-GCAGGGACAGGGACTAGAAAGCAA	AGAAACTTGCCCACAAAGTGAGGA		58-60	6/22	1.75
Np39	$(CA)_{17}$	EU195847	FAM-TATCCCTCTGTCCCCCTTTT	AGAATGGGAAGGTGCTCTTG	1	50-52	7/23	2.5
Np51	$(GT)_{10}$	EU195850	VIC-TTGCAATTACTGCGCTATCC	GTGGATATTCGTGCCCACAT	1	50-52	7./23	2.5
Np78	$(CA)_{10}$	EU195854	NED-GGCTATTCATGGGCATTTTG	CCTCCAATTTTATGCCCATC	2	50-52	6/22	2.5
Np93	$(CA)_3TC(CA)_8G(TA)_6$	EU195855	FAM-TGGATGTGTGATCTGACTTGAA	GGTCAGAGGATGGGAAAGGT	2	50-52	6/22	2.5
Np140	$(GT)_{17}$	EU195848	FAM-TGCTCAAGGAGGATGTTTCC	GCATTGCAACAGTCTGGAAC	3	50-52	6/22	2.5
Np141	$(CA)_{18}$	EU195856	FAM-GGAAGCCAATGAAAGCTCAG	ACACATTTGCTGTGGTCTGG	4	51-53	6/22	2
Np151	$(CA)_9$	EU195843	PET-AATGAGGAAGGAACGTCGTG	GGTTTTGGGGTTGGGTTTT		53-55	10/28	1.75
Np155	$(CA)_{11}$	EU195838	FAM-TTATCACAAAGCCCTCTTGCACAC	ATAGCCACCGAGGAATGCAAAGAG	5	52-54	6/22	1.75
Np163	$(GT)_6TT(GT)_8$	EU195839	FAM-ACCATTCCTTAAGATTGAGAACAC	CATGCAGGACAGGAAAACAAATAG	6	53-55	6/22	1.75
Np166	$(CA)_{11}$	EU195840	FAM-TGCAGTCAAACAGAGTAAAAGG	CTAGCTCCACACTGAGACACAA	5	52-54	6/22	1.75
Np229	$(CTAT)_5CTAC(CTAT)_4$	EU195844	NED-AGGCACCTCACTGACACGTA	AACAAAAATCCCGTATCACGA	6	53-55	7/24	1.75
Np238	$(GT)_{11}$	EU195841	FAM-TATTGGTGTGTCTGCGTG	TGGCCACCTTTTGTAAACAC	4	51-53	7/25	2
Np244	$(GT)_{17}$	EU195842	PET-ACAACACAAGAATACCCTGTCTG	AGCTCTCGAACTGGGACTGA		52-54	6/22	1.75
Np249	$(GT)_{12}$	EU195858	PET-TTTCCCCTTCCTTTCTCCAC	CCGACAGGGAGAGACAAATC	1	50-52	7./23	2.5
Np257	(TA)4$(TG)_8$(TA)2	EU195851	PET-GATTGTCAGGGGTTGCTCAT	CTAGCGCCATTTGTGAAGGT		50-52	6/22	2
Np259	$(TG)_{10}$	EU195852	FAMAAAATGAGTGAAAATAACAAGTAGC	CTGGAAGAGGTCTGCATTAAAA	3	50-52	6/22	2.5
Np296	$(GT)_{14}$	EU195845	FAM-CATACGCCTCTTCCTTCTGC	CCATGGCCAGCTATTGATCT	5	52-54	6/22	1.75

Table 2 Summary of the cross-subspecies amplifications of Egyptian vulture specific microsatellite markers tested on one population from the Navarra (North Iberian Peninsula, *N. percnopterus percnopterus*), and the population from Canary Island (Fuerteventura, *N. percnopterus majorensis*). Number of alleles (NA), size of the PCR product amplified (size range), observed Heterozygosity (H_o), expected Heterozygosity (H_e) and inbreeding coefficient (F_{IS}).

	N. percnopterus percnopterus (N=30)				*N. percnopterus majorensis (N=30)*			
Locus	**Na**	**Size range (pb)**	**H_o**	**H_e**	**F_{IS}**	**Na**	**Size range (pb)**	**H_o**	**H_e**	**F_{IS}**
Np38	2	313-315	0.033	0.033	-0.017	2	313-315	0.100	0.095	-0.053
Np39	11	276-310	0.900	0.891	-0.011	9	276-304	0.724	0.787	0.080
Np51	4	143-155	0.233	0.215	-0.085	2	143-153	0.133	0.124	-0.071
Np78	5	307-316	0.633	0.514	-0.232	3	313-316	0.643	0.536	-0.199
Np93	4	242-248	0.767	0.653	-0.174	3	242-248	0.310	0.344	0.098
Np140	3	396-400	0.500	0.506	0.012	3	396-400	0.333	0.459	0.274
Np141	4	290-300	0.552	0.666	0.172	2	298-299	0.261	0.287	0.092
Np151	2	132-136	0.100	0.095	-0.053	2	132-136	0.233	0.206	-0.132
Np155	7	349-355	0.700	0.653	-0.071	6	350-355	0.667	0.657	-0.014
Np163	4	228-236	0.467	0.568	0.178	6	226-236	0.600	0.689	0.129
Np166	6	190-200	0.567	0.779*	0.273	6	190-200	0.433	0.749*	0.422
Np229	7	171-187	0.900	0.783	-0.149	5	171-183	0.767	0.726	-0.056
Np238	5	150-164	0.233	0.534*	0.563	3	150-162	0.500	0.649	0.230
Np244	5	170-180	0.467	0.654	0.286	3	172-176	0.276	0.499*	0.447
Np249	4	237-245	0.400	0.512*	0.218	5	235-245	0.767	0.735	-0.043
Np257	2	108-110	0.033	0.033	-0.017	1	108			
Np259	3	123-129	0.600	0.660	0.091	3	123-129	0.233	0.263	0.112
Np296	7	168-188	0.700	0.728	0.038	7	172-188	0.897	0.768	-0.167

*Significant heterozygote deficits in exact test of Hardy-Weinberg equilibrium after correction for multiple tests.



Satellite image of the Northwest Africa coasts and the Canarian Archipelago. An example of a trans-Saharan migration route overlapped on Image Aqua MODIS 2004/03/03 at 14:15 UTC, (http://earthobservatory.nasa.gov/) is indicated with black arrows. Note that the return route matched strong east winds from the Sahara and consequently the route was displaced to the west, very close to the Canary Islands.

La ruta migratoria trans-sahariana de un individuo Ibérico se muestra en esta foto solapada sobre la imagen satélite de la costa occidental Africana. Los vientos orientales predominantes parecen desplazar la ruta seguida por este alimoche en su regreso a la Península Ibérica, lo que hace que pase muy cerca de las Islas Canarias.

CHAPTER TWO

Evidence of Connectivity between Continental and Differentiated Insular Populations in a Highly Mobile Species

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Running title: Genetic flow between island and continental vultures

Conectividad entre el continente y las poblaciones insulares diferenciadas: implicaciones para la conservación

Resumen

Las poblaciones insulares (oceánicas) son más vulnerables a los efectos asociados a los tamaños reducidos de población, tales como la deriva génica y la consecuente pérdida de diversidad genética. Estás poblaciones, generalmente aisladas del continente y consecuentemente diferenciadas, son tradicionalmente consideradas y gestionadas como unidades independientes de conservación. Sin embargo, en el caso de especies que cuentan con una considerable capacidad dispersiva, dichas poblaciones pueden recibir inmigrantes provenientes del continente. Esta inmigración, aunque ocasional, jugaría un papel esencial al amortiguar los efectos mencionados (deriva génica) y disminuir de ese modo los riesgos asociados a la endogamia, así como al aumentar su potencial evolutivo. En este segundo capítulo se analiza la estructura de poblaciones y la existencia de conectividad entre el continente y las poblaciones insulares del *Alimoche en el Paleártico occidental*. Finalmente, se discuten las consecuencias en términos de conservación. Los resultados de este trabajo indican que las poblaciones insulares están genéticamente diferenciadas pero pueden recibir inmigrantes ocasionales provenientes del continente. Este dato viene apoyado además por la similitud en los niveles de diversidad genética observados entre los dos tipos de poblaciones (islas y continente). Los resultados demuestran que, a pesar de que se hayan dado procesos de diferenciación, las poblaciones insulares de especies con alta movilidad pueden mantenerse conectadas al continente. De este modo, los programas de conservación deben tener en cuenta la existencia de esas conexiones y gestionar las islas como unidades integradas dentro de redes intra-específicas interdependientes.

Abstract

Aim Genetically differentiated insular populations are candidates for independent units for conservation. However, occasional immigration to reduced island populations may occur and potentially have important consequences in their future viability and evolutionary potential. In this study we investigate the conservation implications of population structure and connectivity of insular and continental populations of a migratory raptor as determined using genetic tools and satellite tracking.
Location Western European populations in the Iberian Peninsula and two insular populations in the Mediterranean Sea (Balearic Islands) and Atlantic Ocean (Canary Islands).
Methods We genotyped 22 microsatellite loci in 96 Egyptian vultures (*Neophron percnopterus*) from the Iberian Peninsula, 36 from Menorca (Balearic archipelago) and 242 (85% of the current population) from Fuerteventura (Canary Islands). We analyzed genetic variation to estimate structure, gene flow, genetic diversity, effective size and recent de
mographic history of the populations. Additionally, nineteen vultures were marked with satellite transmitters to track their migration routes.
Results Insular populations were genetically differentiated from the mainland's. We detected immigration in the insular populations and within the continental counterpart. We found similar levels of genetic variability between the continent and the islands, and a bottleneck analysis indicated recent sharp population declines in both archipelagos but not on the continent.
Main conclusions Our study provides evidence that, in spite of significant differentiation, insular populations of highly mobile species may remain connected with the mainland. Conservation programs should take into account population connectivity and integrate differentiated units of management within complex units of conservation that can best maintain processes and potential for evolutionary change.

Introduction

Understanding population structure and connectivity is crucial for determining units of management for wildlife conservation programs (Moritz, 1994; Saccheri *et al.*, 1998; Segelbacher & Storch, 2002; Schtickzelle *et al.*, 2005; Palsböl *et al.*, 2006; Anderson *et al.*, 2009). Understanding processes structuring populations in environments comprised of discrete spatial units is especially challenging (Haila, 1990). The theory of island biogeography (MacArthur & Wilson, 1967) offers a conceptual framework for the study of differentiated entities. Islands are viewed as dynamic units where immigration and extinction rates occur as functions of island area and isolation. Island isolation is thought to be subject to different spatial scales (Haila, 1990), which implies that immigration-extinction dynamics of geographically isolated demes may not be independent of mainland populations, especially for species with high dispersal capabilities (Whittaker & Fernández-Palacios, 2007).

Insular populations are naturally vulnerable to demographic, environmental and genetic stochastic factors associated with small populations (Pimm *et al.*, 1988, Frankham, 1995). An inevitable consequence of small population size is the loss of genetic diversity due to genetic drift. Drift may eventually result in inbreeding depression, which also may affect the long-term persistence of a population (e.g. Madsen *et al.*, 1996, Lacy, 1997, Acevedo-Whitehouse *et al.*, 2003, Liberg *et al.*, 2005). Accordingly, higher extinction rates have been described in species endemic to islands (Frankham, 1998).

Due to the vulnerability of island populations, their isolation and their phenotypic and genotypic differentiation, insular demes have been traditionally considered as evolutionarily significant units and consequently independent units of management (Moritz, 1994; Palsbøll *et al.*, 2007; Whittaker & Fernández-Palacios, 2007). However, island populations of

highly mobile species may receive migrant individuals from the mainland that may buffer the effects of genetic drift (Westemeier *et al.*, 1998, Madsen *et al.*, 1999; Frankham, 2002; Keller *et al.*, 2002; Marr *et al.*, 2002; Vilà *et al.*, 2003; Hogg *et al.*, 2006; Ortego *et al.*, 2008). The current study addresses the issue of connectivity between continental and insular populations by integrating genetic and satellite tracking information. These approaches are complementary and the number of studies combining them is increasingly frequent in ecology (Bethke & Taylor, 1996; Paetkau *et al.*, 1999; Taylor *et al.*, 2001; Mauritzen *et al.*, 2002; Dearborn *et al.*, 2007; Boulet *et al.*, 2007). While genetic tools provide data on genetic structure, levels of migration among geographically separated populations, genetic diversity and demographic history (Schwartz *et al.*, 2007; Haig, 1998); such methods cannot fully identify specific mechanisms that maintain connectivity among groups. In this sense, satellite tracking can reliably determine the geographic location of individuals throughout their annual cycle and thus allow estimation of the potential for gene flow among locations.

In the present study, we test whether insular populations of a migratory raptor are connected to their continental counterparts, even when they are phenotypically differentiated. We employ 22 neutral genetic markers (microsatellites) and satellite tracking data to determine the genetic structure and migration of insular and continental populations. Our study model is a long-lived, highly philopatric, territorial (Grande, 2006) and migratory scavenger, the Egyptian vulture (*Neophron percnopterus*). This medium-sized (2kg) vulture inhabits dry areas of Europe, Asia and Africa (Fig.1). It is one of the few large raptors able to colonize oceanic islands as it has done in the Atlantic Ocean and the Mediterranean and Arabic seas, although many of these populations are extinct (Levy, 1996; Sarà *et al.*, 2009; Gangoso *et al.*, 2006). Breeding birds maintain exclusive breeding territories but regularly gather in large numbers at feeding sites and communal roosts (Cramp &

Simmons, 1980, Donázar & Ceballos, 1990). Long-term population studies have shown a low level of natal dispersal, with a median of 20 km (range 0-150 km; being slightly higher for females, Grande *et al.*, 2009). Exceptional dispersals of up to 550 km have been detected between some western European populations (Elorriaga *et al.*, 2009). After recruitment into breeding territories, Egyptian vultures show a low rate of breeding dispersal (less than 5% annually) and an average annual survival of 0.9 for adult birds. The generation time for the species (average ages at which females give birth to offspring (Ricklefs & Miller, 2000)) has been estimated to be 13 years (Grande, 2006, Grande *et al.*, 2009, authors unpublished).

Despite its broad distribution (Fig.1), it is estimated that only 30000 to 40000 mature individuals survive in the world and the species is considered "Endangered" (BirdLife International, 2008). In Europe, the Iberian Peninsula (Iberia) holds the bulk of the reproductive individuals but there it has also declined precipitously in recent decades, with a decrease of up to 70% in some regions during the 80's (BirdLife International, 2008). This vulture has also suffered drastic declines on all islands including the two main insular populations remaining in Western Europe: Menorca and the Canary Islands. In the former, the number of breeding pairs was estimated to be 41 in 2002, a decline of 20% in about 10 years (De Pablo, 2002). In the Canary Islands, the population has been described as a differentiated subspecies (*N. p. majorensis*) (Donázar *et al.*, 2002a), and while abundant in the past (Martín, 1987) it has disappeared from most of the islands over the last few decades (Donázar *et al.*, 2002b, Palacios, 2004). At present, the species is found mostly on Fuerteventura (the southeastern-most island) where it has been intensively monitored over the last twelve years (mean of 30 breeding territories/year, SD=6.4). Between two and four breeding pairs were observed every year on the closest island (Lanzarote). The population size for the

Canary archipelago was estimated at about 200 birds in 2009 (authors' unpublished data).

We address whether: i) western Palearctic populations of this species are connected, both within the continent and with respect to the insular locations, ii) the levels of genetic diversity and effective population sizes are lower in insular than mainland populations, and iii) the drastic declines in recent decades of all populations have resulted in population bottlenecks.

Methods

Sample collection

This study was based on samples from Iberia, Menorca (Balearic Islands) and Fuerteventura (Canary Islands) (Fig. 1). Within Iberia we considered three different populations, previously described by Carrete *et al.*, (2009); Northern, with two subpopulations (Navarre and Aragon), Central (Segovia) and Southern (Cadiz). Birds were captured, ringed and sampled between 1995 and 2000 in the Iberian Peninsula (Navarre; n=40, Aragon; n=19, Cadiz; n=22, Segovia; n=15), between 1998 and 2007 in Fuerteventura (n=242) and from 1998 to 2002 in Menorca (n=36). Fledglings were captured in their nests and adult and immature birds were captured with cannon nets at supplementary feeding sites. Birds were aged by plumage (Cramp & Simmons, 1980).

Genetic analysis

DNA extractions were performed using a standard phenol-chloroform extraction (Sambrook *et al.*, 1989). Bird's sex was determined by polymerase chain reaction (PCR) with primers 2550F and 2718R (Fridolfsson & Ellegren, 1999) on DNA extracts from blood samples. All individuals were genotyped using five

non-specific (Gautschi *et al.*, 2000) plus 17 species-specific microsatellite loci (Agudo *et al.*, 2008). All population analyses were carried out with a sub-sample of individuals from Fuerteventura (N=45) to avoid bias due to differences in sample size. Deviations from Hardy-Weinberg and linkage equilibrium were tested using GENEPOP 3.4 (Raymond & Rousset, 1995) followed by sequential Bonferroni correction for multiple comparisons (Rice, 1989).

Population structure

Population structure was measured using two approaches. First, pair-wise population differentiation based on F_{ST} (Weir & Cockerham, 1984, significance tested by 10000 permutations) and a factorial correspondence analysis (FCA) calculated using GENETIX 4.03 (Belkhir *et al.*, 2004). Second, we employed the Bayesian method of Pritchard *et al.*, (2000) and Falush *et al.*, (2003) implemented in STRUCTURE (version 2.1) to cluster individuals into respective subpopulations and reveal patterns of gene flow across populations. This program employs a Bayesian clustering to identify the most likely number of populations (K) that are in Hardy-Weinberg and linkage equilibrium assuming no *a priori* structure.

The first step in the analysis involved estimating the number of subpopulations (K). For that, we first investigated the most likely K considering all sampled populations running five independent simulations of K=1-7. Then, we ran another five independent simulations excluding the insular individuals (K=1-5) in order to infer cryptic population structure within the mainland. All simulations were run under standard parameters in the admixture model with the option of correlated allele frequencies at 30000 iterations of burn-in, followed by 100000 iterations. In both cases the most likely value of K was chosen using the ΔK

statistic, based on the rate of change between successive *K* values, as recommended by Evanno *et al.*, (2005).

The second step of the analysis involved assigning individuals to each of the sampled subpopulations. The clustering approach used in STRUCTURE when investigating population structure can be incorporated when attempting to determine which individuals are not residents of their sampled population. When the "usepopinfo" option of STRUCTURE is employed, the program assumes an initially high probability that each individual is a resident of its sampling locality. Using prior population information allows the program to calculate posterior probabilities that individuals belong to their sampled locality/cluster. To infer migrants to the insular populations, we first ran the program with all Canary Island (n=242) and all Iberian individuals as one population. Then, to detect migration among the continental demes, we ran the program with only the Iberian birds. For the analyses within Iberia, and given that Aragon and Segovia did not show a significant differentiation based on the F_{ST} values, we grouped these two locations in a single cluster. We defined individuals with *q*-values from 0.8 to 0.2 as potentially admixed (Bergl & Vigilant, 2007; Lecis *et al.*, 2006; Vähä & Primmer, 2006). Burn-in and run length were the same as for runs without a priori population information.

Finally, to estimate migrants in STRUCTURE we performed an exclusion test implemented in GENECLASS 2.0 (Paetkau *et al.*, 2004; Piry *et al.*, 2004). Again we performed the analyses clustering the individuals from Aragon and Segovia. We selected the 'detect migrants' function explicitly designed to identify first generation migrants, i.e. individuals born in a population other than the one in which they were sampled. This program uses likelihood-based statistics, in combination with resampling methods, to calculate probabilities that individuals are first generation migrants. We used two different likelihood-based test statistics to identify migrant individuals. Lh, the likelihood of finding a given

individual in the population in which it was sampled, is the most appropriate statistic to use when all potential source populations have not been sampled (Paetkau *et al.*, 2004; Piry *et al.*, 2004). However, Lh lacks power when compared to other estimators and thus we also used Lh/Lmax, the ratio of Lh to the greatest likelihood among all sampled populations (Paetkau *et al.*, 2004), which has greater power. We employed the Bayesian criterion of Rannala & Mountain (1997) in combination with the resampling method of Paetkau *et al.*, (2004) to determine the critical value of the test statistic (Lh or Lh/Lmax) beyond which individuals were assumed to be migrants. We selected an alpha level of 0.01 to determine critical values (Paetkau *et al.*, 2004).

In addition, to test if the rate of gene flow within the mainland populations was influenced by geographic distance, we related genetic and geographic distances among the four locations (Navarre, Aragon, Segovia and Cadiz). We determined the geographical UTM coordinates of the approximate distribution midpoints for each of the sampled populations and we then calculated pair-wise Euclidean distances between sites. A Mantel test with 10000 random permutations to test significance was performed between the matrix of pairwise genetic differentiation between populations ($F_{ST}/(1 - F_{ST})$, Rousset, 1997), and the matrix of the geographic distance. The analyses were performed with IBDWS (Jensen *et al.*, 2005).

Genetic diversity, bottleneck analysis and effective population size estimates

The genetic diversity of populations was estimated using GENALEX version 6 (Peakall & Smouse, 2005) and differences among populations were compared using Wilcoxon sign-rank tests. In order to detect recent population declines we used BOTTLENECK (Cournet & Luikart, 1996). This method tests for a heterozygosity excess relative to the number of alleles. It is based

on the theoretical expectation that in bottlenecked populations the number of alleles is lost more rapidly than heterozygosity decreases. We used the two-phase mutation model (TPM) allowing the default 30% multi-step mutation events, as recommended by the authors of this software in the case of microsatellite data. Effective population sizes were calculated by the linkage disequilibrium method using NEESTIMATOR (Peel *et al.*, 2004).

Satellite tracking

To test whether seasonal migratory movements favour the arrival of individuals to the islands, nineteen Iberian Egyptian vultures were equipped with Platform terminal transmitters (PTT) from 2002 to 2004. Eight of these birds were fledglings captured at nests in Cadiz, while four immature individuals of one-, two- and three-years-old and one fledgling were capture at supplementary feeding sites in central Iberia (Ciudad Real). The remainder of birds (one fledgling, four adults and two immature birds one-year-old) were captured at supplementary feeding sites in northern Iberian (Basque Country, Navarre and Aragon). Three individuals were not sexed and there were twelve females and four males.

We used PTTs of two different types: 12 battery powered (PTT100, weight 45 g from Microwave Telemetry Inc., USA) and five 40-42 g solar PTTs (North Star Science and Technology). They were set to an 8-h on/120-h off duty cycle. The full transmitter equipment never exceeded 3% of the mean body mass of full-grown Iberian birds (1965 g, Donázar *et al.*, 2002a). Locations were determined by means of the Argos satellite system (http://www.argosinc.com/). Argos provides up to seven classes of locations with different degrees of accuracy. We conservatively used only those locations assigned to classes 1, 2 and 3, i.e. with accuracy < 1000 m (see e.g. Cadahía *et al.*, 2005). We obtained a

total of 2356 locations with this precision. To visualize locations we employed ARCVIEW 3.2 (Hooge & Eichenlaub, 1997), and as a cartographic base we used Esri Data and Maps-Digital Chart of the World (http://www.maproom.psu.edu/dcw), Global Land Cover 2000 (Hhttp://wwwgvmH. jrc.it/glc2000/), Gtopo (http://edc.usgs.gov/products/elevation/gtopo30/gtopo30.html), and Orthorectified Lansat Enhaced Thematic Mapper (https://zulu.ssc.nasa.gov/mrsid/).

Results

Tests of linkage disequilibrium and null alleles

Significant deviations from Hardy-Weinberg equilibrium in the form of heterozygote deficits were present in loci Np238 and Np244 in all populations. These deficiencies were most likely due to non-amplifying alleles ("null alleles") at both markers and thus they were excluded from further analyses. Exact tests for genotypic linkage disequilibrium (LD) confirmed the absence of physical linkage at most loci. However, some pairs of loci for a limited number of populations appeared to have significant LD (np93 and np140 in Navarre and np244 and bv9 in Menorca; $p<0.05$ after Bonferroni correction). Because no significant LD for these pairs of loci was detected elsewhere, we concluded that these loci can be treated as independent markers.

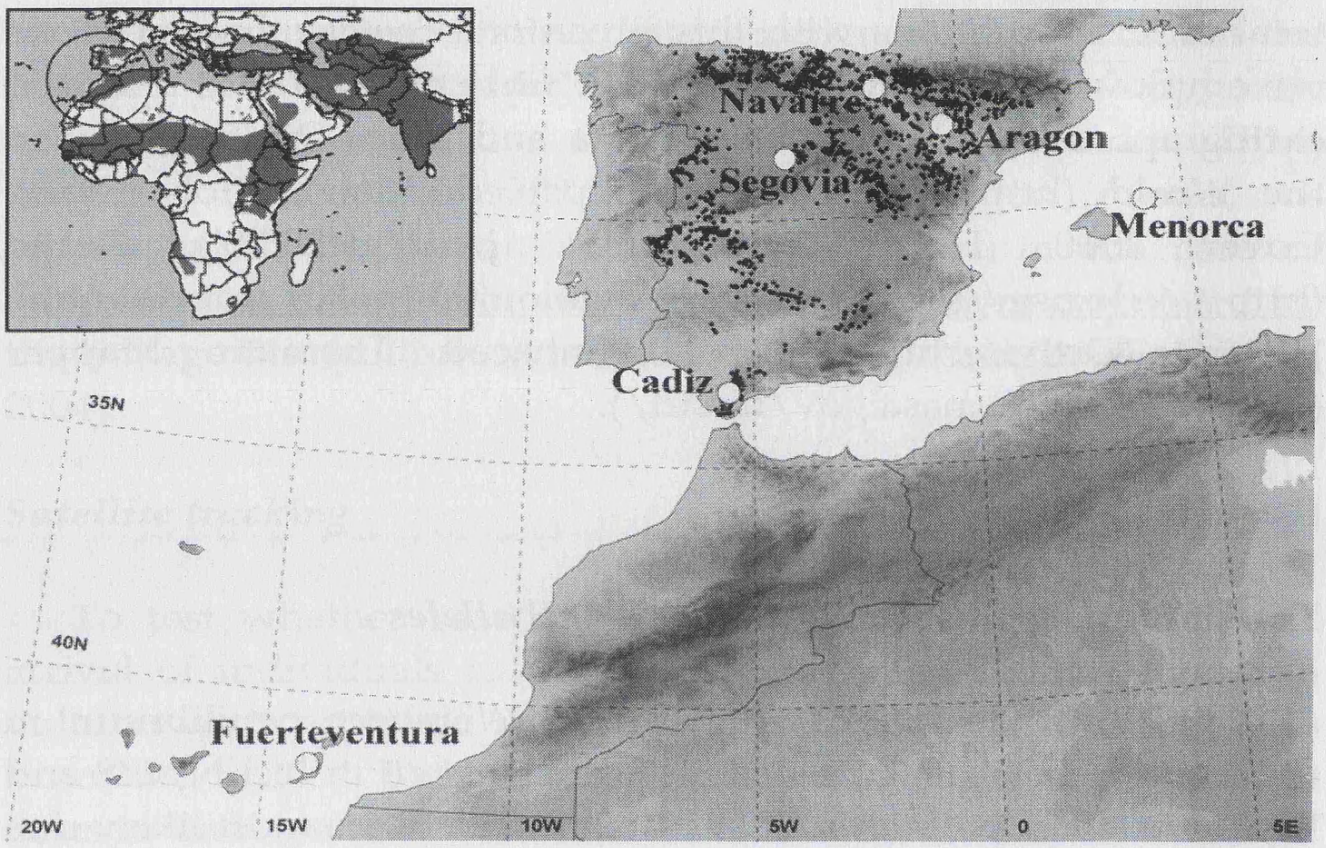

Fig 1 Distribution area of the species in the Iberian Peninsula (indicated by black spots that represent occupied territories in 2000 (Del Moral 2009)) and sampling locations for this study (white spots). The map in the upper left-hand corner represents the global species' distribution. The orthographic map projection was employed.

Population differentiation

Genetic differentiation across all populations measured as F_{ST} indicated that island populations are clearly differentiated from those on the mainland. All pairwise F_{ST} values were significant except between the populations from Segovia and Aragon, and the highest genetic distance was found between the two island populations (Table 1). The factorial correspondence analysis (FCA) was consistent with these results and showed the presence of one bird from Fuerteventura with a genotype compatible with continental individuals (Fig. 2).

Calculation of ΔK from the STRUCTURE output produced a modal value of the statistic at K=3 (Fig. 3 and 4). The height of the modal values of ΔK indicates the strength of the population subdivision signal (Evanno *et al.*, 2005), suggesting deep subdivision at K=3. However, examination of Ln $P(X \mid K)$ values from the program suggested a level of subdivision at K=4 indicating a partial structure within the continental populations (Fig. 4). In fact, when excluding the insular populations in the STRUCTURE analysis the ΔK statistic suggested a population subdivision at K=2 (northern plus central and southern) (Figs. 3 and 4).

All runs at K=3 produced identical clustering solutions with similar values of cluster membership q for all individuals within localities. Almost all individuals from the islands were assigned to their respective geographic populations with q>0.85, and vultures from the Iberian Peninsula were assigned to a single cluster with q>0.84 (Table 2). Results for K=4 clustered most of the southern Iberian individuals (from Cadiz) into a distinct cluster with a high probability (q=0.8).

Table 1 Pairwise F_{ST} values between populations (CAD: Cadiz, ARA: Aragon, NAV: Navarre, SEG: Segovia, MEN: Menorca and FV: Fuerteventura).

	CAD	ARA	NAV	SEG	MEN	FV
CAD	-					
ARA	0.025*	-				
NAV	0.03**	0.016*	-			
SEG	0.017*	0.011	0.028*	-		
MEN	0.095**	0.078**	0.085**	0.079**	-	
FV	0.114**	0.103**	0.104**	0.11**	0.144**	-

*p<0.05 **p<0.01

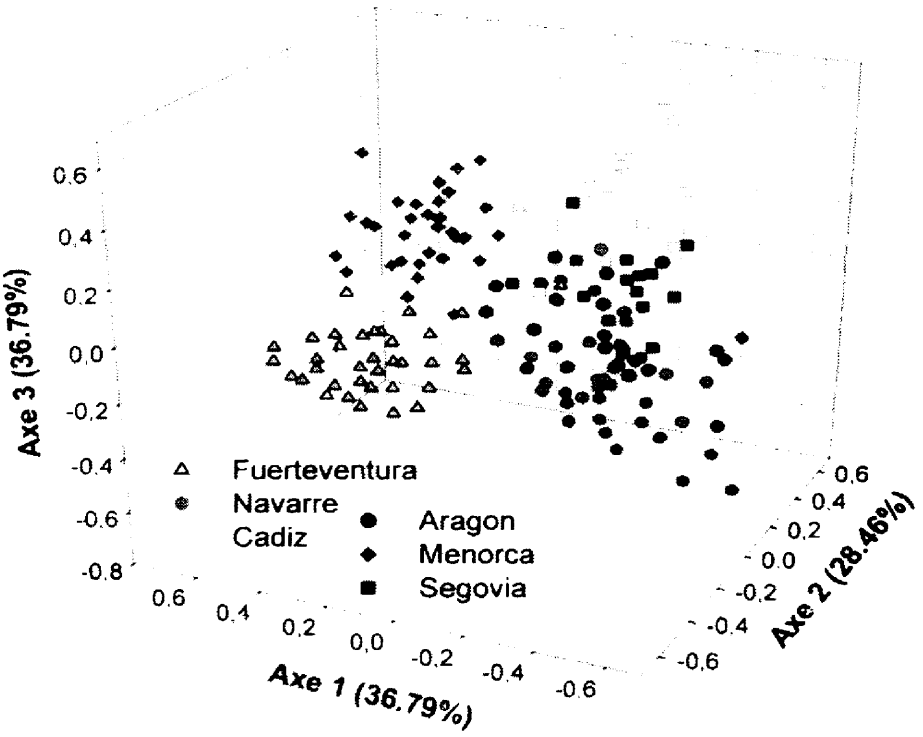

Fig 2 Scatter plot of the factorial correspondence analysis based on the allele frequencies of 22 microsatellite loci. The two insular and four continental populations are indicated. Suggested Iberian migrants found in Fuerteventura (based on the analysis in STRUCTURE and GENECLASS) are marked with a circle.

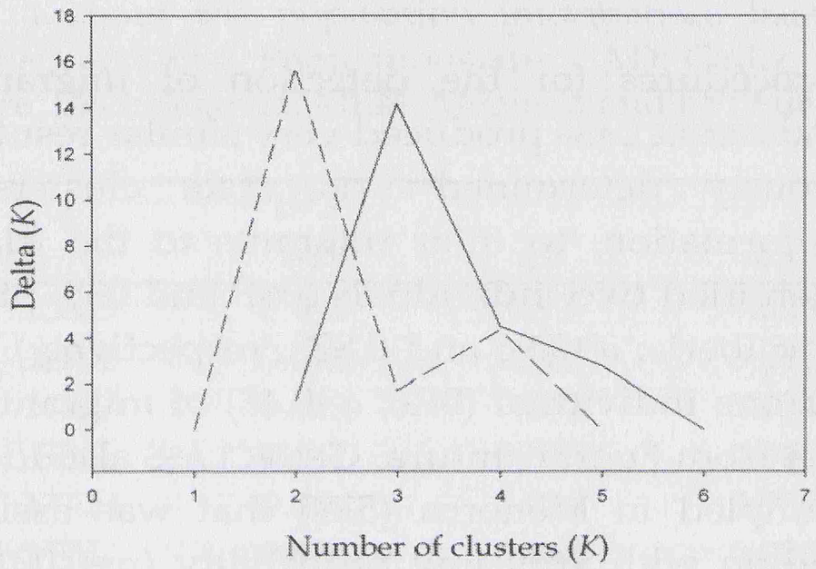

Fig 3 Rate of change in log-likelihood values (delta (*K*)) for estimated number of populations (Evanno *et al.*, 2005).The maximum delta (*K*) indicates the most likely number of clusters: within the Iberian Peninsula (excluding the insular demes, dashed line) and for all populations (solid line)

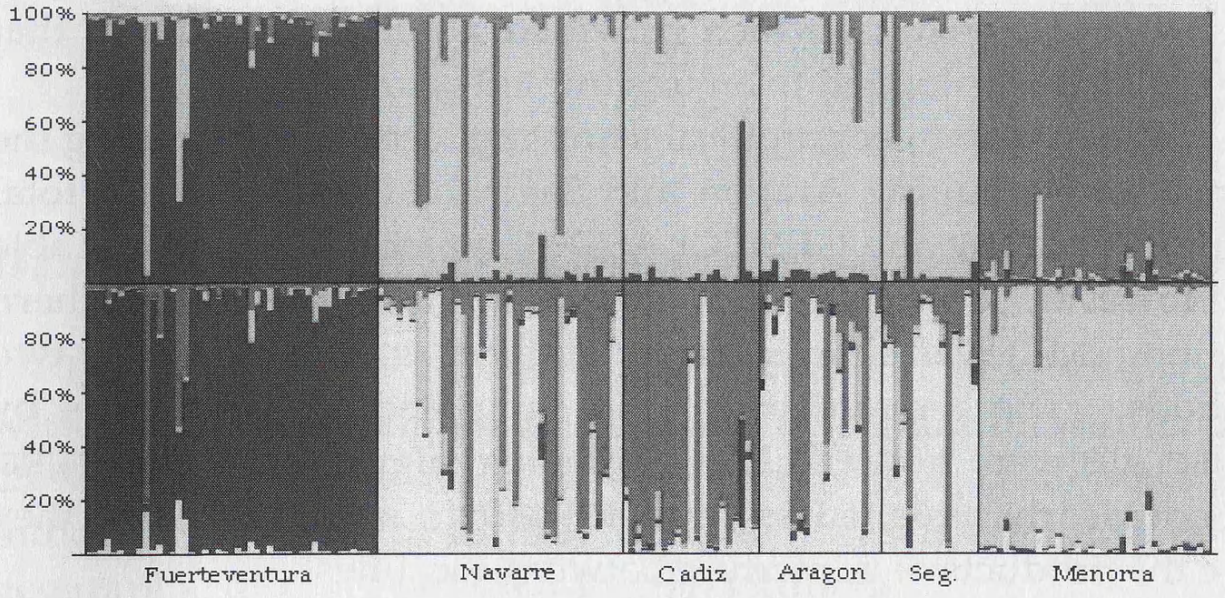

Fig 4 Clustering analysis in STRUCTURE, without prior population information, based on sample location and from *K*=3 (above) and *K*=4 (below). Each individual is represented as a colour bar, where the amount of each colour indicates the proportion of each inferred cluster. Geographic populations of origin are indicated.

Detection of migrants

The procedures for the detection of migrants in both STRUCTURE and GENECLASS produced very similar results (Table 3). Using previously determined STRUCTURE clusters as prior population information, to infer migrants to the islands (K=3), STRUCTURE identified two individuals (06P and 035; probability of membership to Iberia: q=0.96 and 0.823, respectively) as potential migrants, and one individual (0R6; q=0.48) of migrant ancestry in the population from Fuerteventura. GENECLASS also identified one individual sampled in Menorca (539) that was assigned to its geographic origin with very low probability (p=0.001) (Table 3). This migrant was identified with Lh which suggests that this bird represents a migrant from an unsampled area. To corroborate this finding we performed an exclusion test in GENECLASS including the individual from Menorca (539) in the two other populations: Iberia and Fuerteventura. We obtained very low assignment probabilities for all localities (Fuerteventura p<0.001; Iberia p=0.02 and Menorca p=0.001) which provided additional evidence that this individual belonged to an unsampled source.

When using geographical sampling localities (excluding the islands and grouping Aragon and Segovia) we detected in total (using STRUCTURE and GENECLASS) six potential migrants (Table 3). However, only one individual captured in the southern location (1ML) was significantly identified as migrant by the two methods. The remainders of the suggested migrant birds by GENECLASS were not readily classified as migrants by STRUCTURE, but not clearly assigned as residents either, suggesting that they were the products of admixture between localities.

Table 2 Average proportion of membership in clustering analysis of STRUCUTURE, without the population information, based on sample location for *K*=3 and *K*=4. Populations are: CAD: Cadiz, ARA: Aragon, NAV: Navarre, SEG: Segovia, MEN: Menorca and FV: Fuerteventura.

	k=3		
	***q*1**	***q*2**	***q*3**
CAD	0.048	0.93	0.023
ARA	0.028	0.907	0.065
NAV	0.023	0.843	0.134
SEG	0.041	0.895	0.064
MEN	0.016	0.028	0.956
FV	0.914	0.063	0.023

	k=4			
	***q*1**	***q*2**	***q*3**	***q*4**
CAD	0.802	0.019	0.032	0.147
ARA	0.315	0.051	0.024	0.61
NAV	0.303	0.111	0.02	0.567
SEG	0.269	0.054	0.025	0.651
MEN	0.028	0.933	0.015	0.024
FV	0.058	0.021	0.897	0.025

Isolation by distance within Iberia

The Mantel test between geographic and genetic distances within Iberian subpopulations showed no significant results (Z =-25.97, r = 0.39, one-sided p = 0.35 from 1000 randomizations) indicating that the gene flow observed was not limited by the geographic distance between Iberian locations.

Genetic diversity and inbreeding

Values of genetic diversity are summarized in Table 4. A total of 130 alleles was detected in the 6 populations. Average number

of alleles ranged from 3.9 to 5 (Fuerteventura and Navarre, respectively) and mean allele richness ranged from 2.42 to 3.09 (Fuerteventura and Aragon, respectively). The comparison of genetic diversity among the previously described subpopulations (see above) indicated that the northern-central Iberian subpopulation had a significantly ($p<0.01$) higher average number of alleles (Na=5.7) than the southern one (Na=4.7, p=0.01, Z=2.55) and the two insular demes, for which we found the lowest values (3.9 and 4.0 for Fuerteventura and Menorca respectively, p=0.001, Z=3.25 in both cases). Subsequently, the number of alleles was significantly higher in the southern location than in the islands (Fuerteventura, p=0.03, Z=2.17 and Menorca, p=0.01, Z=2.43). Mean allele richness was only significantly different between the northern-central subpopulation (Ne=2.99) and Fuerteventura (Ne=2.4) (p=0.03, Z=2.13). Values of expected heterozygosities (H_E) were also significantly higher in the Iberian locations (both northern-central (H_E=0.56) and southern (H_E=0.55) compared to Fuerteventura (H_E=0.47) (p=0.01, Z=2.35 and p=0.04, Z=1.97, respectively) while no significant differences were found between the two insular demes. Population inbreeding coefficients (F_{IS}) were positive in the populations from Cadiz, Segovia and Fuerteventura and negative in the rest, but none of them was significantly different from zero (1000 permutations) (Table 4).

Bottleneck analyses and effective population sizes (N_e)

The observed proportion of heterozygotes was significantly different than expected under mutation-drift equilibrium in the two insular populations analyzed (one tail for H excess: FV, p=0.017; MEN, p=0.005), which indicated that they have suffered a recent bottleneck. We detected partially significant signs of recent population declines in northern locations and no signs of bottleneck in the central and southern locations (one tail for H

excess: NAV, p=0.071; ARA, p=0.065; CAD, p=0.274 and SEG, p=0.153).

The estimations of the effective population sizes were 38.8 (95% CI 36.1-41.7) for Fuerteventura, which closely matched the current number of successfully breeding birds (mean number of breeding pairs during the last 8 years=35), and 34.7 (95% CI 28.9-42.6) for Menorca.

For the Iberian populations, estimations were carried out according to the observed population structure (see below): northern and central locations together *Ne*=128.0 (95% CI 79.4-120.3) and the southern location *Ne*=45.5 (95% CI 33.7-67.1).

Table 3 Results of migrant detection analysis. Individuals significantly identified as potential migrants by at least one of the two approaches (implemented in STRUCTURE and GENECLASS) are shown. Asterisks indicate individuals significantly suggested as migrants by STRUCTURE. Populations are: IBE: all Iberian populations grouped as a single cluster, FV: Fuerteventura, MEN: Menorca, NAV=Navarre, CAD: Cadiz and ARA+SEG: Aragon and Segovia grouped as a single population (based on F_{ST} statistics).

		STRUCTURE	GENECLASS		
ID (ring)	**Origin**	***q* (with pop. information)**	**migrants F_0 (-log (Lhome)**	**Prob. pop. origin Lh/(Lh/Lmax)**	**assigned population (-log (L))**
		K=3 (IBE | FV | MEN)			
06P	FV	0.956 | 0.00 | 0.00*	27.04	0.00/0.00	ARA (19.29)
035	FV	0.823 | 0.00 | 0.00*	19.39	0.017/0.018	ARA (19.35)
0R6	FV	0.479 | 0.474 | 0.00*	17.74	0.037/0.028	CAD (17.37)
539	MEN	0.00 | 0.00 | 0.724	21.59	0.001/0.05	MEN (21.59)
		K=3 (NAV | CAD | ARA+SEG)			
4F9	NAV	0.505 | 0.211 | 0.091	20.76	0.017/0.001	CAD (16.71)
1ML	CAD	0.015 | 0.203 | 0.706*	23.26	0.002/0.00	ARA (15.86)
267	SEG	0.001 | 0.115 | 0.710	23.33	0.004/0.004	CAD (19.85)
258	ARA	0.002 | 0.322 | 0.520	29.09	0.14/0.001	CAD (14.53)
316	ARA	0.076 | 0.00 | 0.767	21.17	0.03/0.001	NAV (16.55)
323	ARA	0.001 | 0.019 | 0.871	19.97	0.04/0.004	CAD (16.75)

Table 4 Genetic diversity as: mean number of alleles (N_A), mean allelic richness (*AR*), number of private alleles (N_P), observed and expected heterozygosities (*Ho, He*), and inbreeding coefficient (F_{IS}) of Egyptian vulture populations (CAD: Cadiz, ARA: Aragon, NAV: Navarre, SEG: Segovia, MEN: Menorca and FV: Fuerteventura).

Pop.	N	Mean N_A	Mean *AR*	N_P	*Ho* (SD)	*He* (SD)	F_{IS} (95% CI)
CAD	22	4.7	2.69	8	0.55 (0.23)	0.55 (0.20)	0.022 (-0.07 to 0.03)
ARA	19	4.8	3.08	4	0.60 (0.27)	0.57 (0.24)	-0.013 (-0.011 to 0.02)
NAV	40	5.0	2.95	4	0.55 (0.24)	0.55 (0.23)	-0.004 (-0.07 to 0.02)
SEG	15	4.3	2.64	3	0.53 (0.23)	0.54 (0.22)	0.03 (-0.08 to 0.07)
MEN	36	4.0	2.59	2	0.53 (0.27)	0.53 (0.24)	-0.024 (-0.09 to 0.02)
FV	45	3.9	2.42	3	0.47 (0.27)	0.48 (0.24)	-0.007 (-0.07 to 0.03)

Satellite tracking

Information obtained from the PTTs indicated that Egyptian vultures flew over the Sahara Desert to spend the winter in the western part of the biogeographic zone known as the Sahel in southern Mauritania. Birds of different age, sex and location of origin followed similar migration routes and wintered in the same area (Fig. 5). In general, autumn movements started at the end of August and birds crossed the Sahara Desert more or less in a straight line from southern Spain to the south of Mauritania (Fig. 5). We could only record spring migratory movements to the Iberian Peninsula in six cases, between the end of February to the end of May. All birds returned to the localities where they were captured. Whereas four birds followed similar routes to those in autumn, in two cases the vultures returned along the coast of West Africa (Fig. 6). None of these birds reached the islands. We did not detect movements of individuals to the eastern Iberian coast or the Balearics.

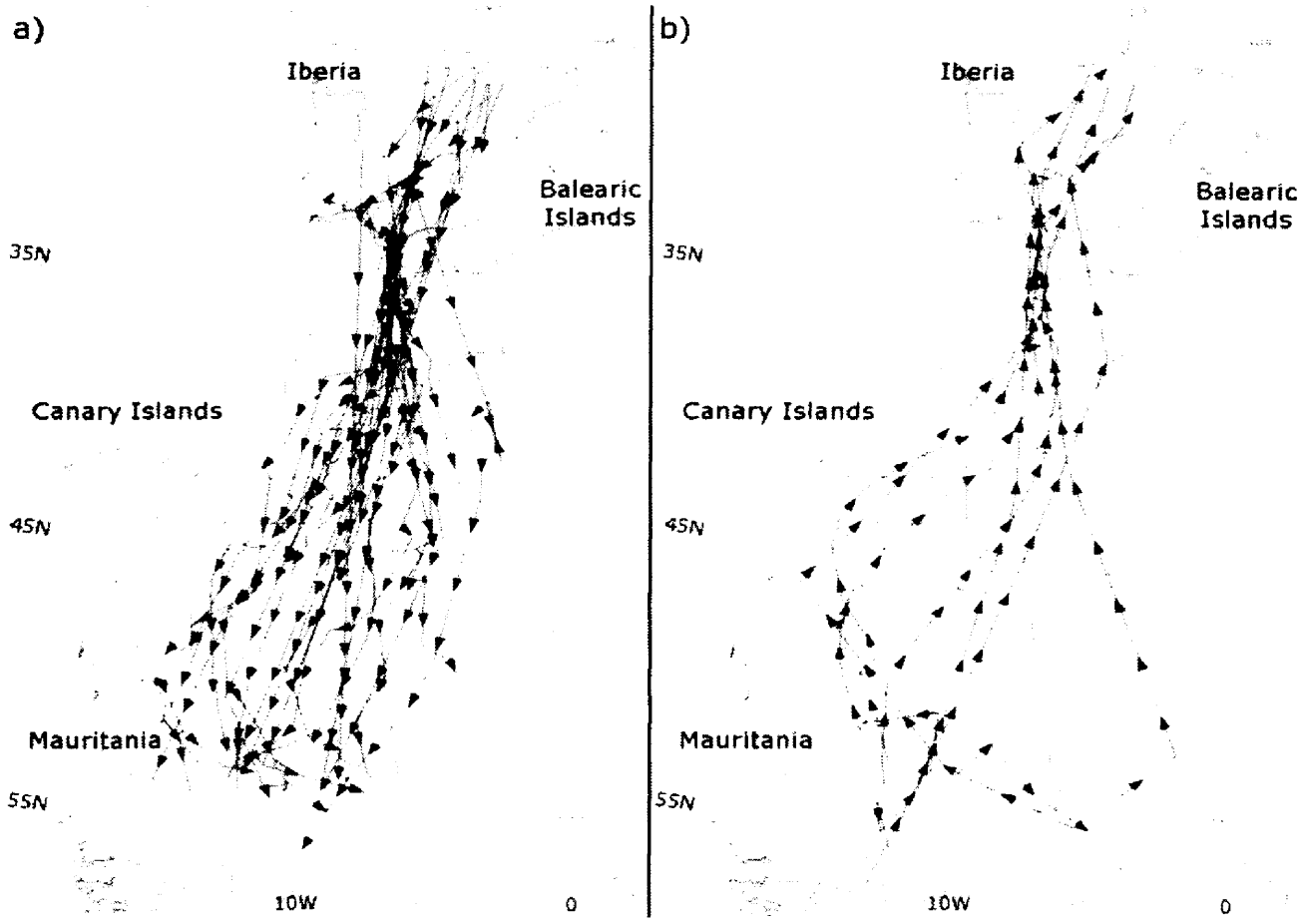

Fig 5 Migratory routes of 19 Egyptian vultures tracked with global positioning system satellite telemetry from the Iberian Peninsula to Africa during (a) autumn and (b) spring migration. The Mollewide map projection was applied.

Discussion

Connectivity between insular and continental populations

We demonstrate that isolated and differentiated insular populations of highly mobile species can be connected with the continent by occasionally receiving immigrants. The genetic, morphologic and ecological differentiation indicate limited gene flow to the Egyptian vultures of the Canary Islands,

demonstrating that insular birds have diverged from the European source population and adapted to the new island environment, giving rise to the present differentiated subspecies (Agudo R. *et al.*, in press; Donázar *et al.*, 2002a). On the other hand, the genetic and satellite data suggest the existence of a certain degree of connectivity between the Canary Islands and Iberia that has not masked the observed differentiation. We found two Iberian immigrants (birds 06P and 035, Table 3) and one bird of mixed ancestry (bird 0R6, Table 3) on Fuerteventura. Even though a correction for multiple tests should have ideally been applied to avoid type I errors, the observed convergence in the results between the two approaches confirms our findings and suggests that, although rare, admixture can occur. This finding substantiates the fact that, like many other trans-Saharan European species (Martín & Lorenzo, 2001), Egyptian vultures occasionally reach the archipelago.

The migration routes obtained by the satellite tracking information agree with previously reported observations in other Iberian and French individuals of this species (Meyburg *et al.*, 2004; García-Ripollés *et al.*, 2010). All vultures from Western Europe follow similar routes across Morocco and Mauritania to overwinter in the Sahel region of Senegal, Mauritania and Mali. Spring migration routes often follow the West African coast and pass close to the Canary archipelago, 95 km away, which is crossable distance by the species (Cramp & Simmons, 1980, and see García-Ripollés *et al.*, 2010). This finding suggests that the European Egyptian vultures can easily reach the islands, as it is undoubtedly evidenced by our genetic information. In fact, other more abundant migratory species that follow similar routes are regularly observed in the archipelago (Martín & Lorenzo, 2001).

Although our information is sparse, a similar scenario may also occur in the Balearic Islands. Our results suggest that gene flow may be limited between the Balearics and Iberia but, on the other hand, they do indicate the presence of a potential migrant

(bird 539, Table 3) from an unsampled population. These are not completely unexpected results. These islands are relatively close to the continent and many migratory birds, including large birds of prey, regularly reach the archipelago (GOB 2004; Bannerman & Bannerman, 1983). We would expect then that the arrival to the Balearics is favoured by migration. Even though the suggested immigrant has not been assigned to any of the sampled Iberian locations, we can not exclude the possibility that it comes from an unsampled area in Iberia or from another European population (Fig. 1).

Within-mainland population structure

Observed pair-wise fixation indexes within Iberia contrast with the values previously reported by Kretzman *et al.,* (2003). In that study the authors did not detect a significant differentiation within the Iberian locations. Although their study provided a baseline for genetically characterising Egyptian vulture populations, it was performed with a low number of heterospecific microsatellite loci and small sample sizes. The use of a larger set of specific markers, larger sample sizes and the Bayesian approach have allowed us to obtain a more accurate characterization of the structure within Iberia. Pair-wise comparison fixation indexes, PCA and Bayesian analyses indicate the existence of a population subdivision within Iberia and suggest the southern deme (Cadiz) as a genetically differentiated entity (Fig. 2 and 4). This location is the most geographically isolated of the studied populations but IBD analysis revealed that its genetic differentiation cannot be explained by isolation. Instead, our results seem to support the recent fragmentation of a formerly continuous distribution of a large, panmictic population (Carrete *et al.,* 2007). All Iberian populations have experienced drastic declines since the 80's, decreasing as much as 70% in some regions (BirdLife International, 2008). These declines are

associated with the high mortality of adults caused by poisoning, collisions with wind power turbines and electric lines and electrocution, and the loss of suitable habitats and food scarcity due to human disturbance (BirdLife International, 2008). Preventive measures should thus be taken to stop the current decline, with special attention to the southern subpopulation, and to facilitate the conservation of populations in Iberia, the main homeland of the European birds.

Genetic diversity

The genetic diversity reported in this study complements results previously obtained for this species using seven non-specific microsatellite loci and conventional population genetics methods (Kretzman *et al.*, 2003). Specifically, Kretzman *et al.*, described more similar levels of H_O in Menorca (0.47) and Iberia (0.37) than with respect to Fuerteventura where genetic diversity was lower (H_O=0.31).

Our results of genetic variability are also comparable to those recently described in other long-lived vulture species (values of H_O based on microsatellites in four other species from Europe and the Middle East ranged from 0.47 to 0.67; Gouar *et al.*, 2008; Arshad *et al.*, 2009).

Despite the progressive decline of the species in its western distribution, our results do not seem to indicate alarmingly low levels of gene diversity. However, with respect to the insular groups, it is worth highlighting the similarities observed. The ratio of heterozygosity between insular and mainland populations (H_{Is}/H_M) was 0.85 for birds from Fuerteventura and 0.95 for Menorca. While the first value is similar to the ratio found by Frankham (1997) in 27 different avian species (0.79), the ratio for Menorca is considerably higher. Accordingly, we did not detect differences in genetic diversity between Menorca and the mainland subpopulations. These similarities contrast with

previous studies (see Frankham, 1997 for a review) and may be partially caused by the existence of gene flow to the insular demes.

The present population declines and the subsequent reduction in the rate of gene flow may have serious consequences on the future genetic health and survival of these threatened insular populations, which also have very low effective population sizes (<50) and are already drastically reduced. In the case of the individuals from the Canary Islands, the detected bottleneck would correspond to the well documented strong decline that occurred over the last few decades. It was caused by the high level of unnatural adult mortality related to human activity including poisoning, lead intoxication and electrocution (Donázar *et al.*, 2002b, Gangoso *et al.*, 2009). To the contrary, the Balearic population is considered to be well preserved, being the densest of the Spanish groups (1 breeding pair/6.7 km^2) (Del Moral, 2009). However, our genetic results indicate that a sharp decline has affected this deme as well, that the past Balearic population was much larger and probably occupied not only Menorca but also other islands of the archipelago (Bannerman & Bannerman, 1983). This suggestion is supported by observations of birds breeding on the island of Mallorca (Adrover, 2005).

An adult Egyptian vulture is trying to break an ostrich egg using a stone; a very characteristic and unique behaviour of this species

Un Alimoche adulto intenta romper un huevo de avestruz empleando una piedra; un comportamiento característico y único de esta especie.

CHAPTER THREE

MHC variation in Insular Populations of the Egyptian vulture: Inferences about the Roles of Genetic Drift and Selection

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Running title: MHC variation in island vultures

Variación en el MHC en dos poblaciones insulares de Alimoche (*Neophron percnopterus*): inferencias acerca de los roles de la deriva génica y la selección

Resumen

Los trabajos enfocados a la genética de la conservación de las poblaciones amenazadas han empleado tradicionalmente marcadores neutrales. Sin embargo, es el estudio de los genes funcionales el que nos puede aportar una información más precisa acerca de la distribución de la variación genética adaptativa y de los posibles efectos negativos relacionados con la pérdida de diversidad genética. Por otro lado,. el estudio comparativo de la distribución de la variabilidad genética entre genes neutrales y funcionales, así como entre poblaciones sujetas a diferentes presiones selectivas, puede ayudarnos a esclarecer cuál es el papel de cada una de las distintas fuerzas evolutivas a la hora de dibujar el perfil genético de las poblaciones. Este es uno de los principales objetivos de la ecología molecular y la biología evolutiva. El problema estriba en que aun hoy, muy pocos genes funcionales han sido caracterizados en las poblaciones naturales. En ese sentido los Genes del Complejo Mayor de Histocompatibilidad (MHC) son una excepción y se convierten en los candidatos perfectos para este tipo de estudios. Por un lado, se encuentran entre los genes funcionales mejor estudiados en el mundo animal. Por otro lado, constituyen un componente esencial del sistema inmunológico de los individuos, por lo que están directamente relacionados con la calidad individual y la supervivencia.

En este capítulo integramos el estudio de la variación neutral (22 microsatélites y un locus mitocondrial) y adaptativa (genes de la clase II β del MHC) en una población continental (Península Ibérica) y dos insulares (Canarias y Baleares) de Alimoche. Nuestros resultados indican valores de diversidad genética menores en las islas que en el continente. En cuanto a los procesos evolutivos responsables de los patrones de variación observados, vemos por un lado, que la deriva génica ha predominado como fuerza evolutiva histórica en las islas. Sin embargo, las frecuencias que los distintos grupos de ligamiento de los genes del MHC presentan en las poblaciones insulares, nos sugieren la existencia de fuerzas de selección diversificadora a más corto plazo. El resultado más interesante de este trabajo es, no obstante, la aparente co-evolución entre las dos duplicaciones amplificadas del gen. Esta co-evolución ha podido permitir que pares de alelos divergentes se mantengan unidos por estrechas fuerzas de ligamiento en las islas. Este mecanismo puede así haber contrarestado la pérdida de diversidad sufrida en las poblaciones isleñas, maximizando las capacidades de reconocimiento antígeno cuando la diversidad se ha visto reducida y promoviendo la co-segregación de las combinaciones de alelos más eficientes a la hora de responder a las comunidades de patógenos locales.

Abstract

Insular populations have attracted the attention of evolutionary biologists because of their morphological and ecological peculiarities with respect to their mainland counterparts. Founder effects and genetic drift are known to distribute neutral genetic variability in these demes. However, elucidating whether these evolutionary forces have also shaped adaptive variation is crucial to evaluate the real impact of reduced genetic variation in small populations. Genes of the Major Histocompatibility Complex (MHC) are classical examples of evolutionarily relevant loci because of their well-known role in pathogen confrontation and clearance. In this study, we aim to disentangle the partial roles of genetic drift and natural selection in the spatial distribution of MHC variation in insular populations. To this end, we integrate the study of neutral (22 microsatellites and one mtDNA locus) and MHC class II variation in one mainland (Iberia) and two insular populations (Fuerteventura and Menorca) of the endangered Egyptian vulture *(Neophron percnopterus)*. Overall, the distribution of the frequencies of individual MHC alleles (N=17 alleles from two class II B *l*oci) does not significantly depart from neutral expectations, which indicates a prominent role for genetic drift over selection. However, our results point towards an interesting co-evolution of gene duplicates that maintains different pairs of divergent alleles in strong linkage disequilibrium on islands. We hypothesize that the co-evolution of genes may counteract the loss of genetic diversity in insular demes, maximize antigen recognition capabilities when gene diversity is reduced, and promote the co-segregation of the most efficient allele combinations to cope with local pathogen communities.

Introduction

Reduced and bottlenecked populations are exposed to the effects of genetic drift and loss of genetic diversity. Loss of genetic diversity may increase the risk of extinction of small populations because of inbreeding depression and decreased adaptive potential (Mills, 2006). Understanding the mechanism determining the levels of genetic variation in reduced and fragmented populations is therefore essential for their conservation (Frankham *et al.* 2002). Islands are a particular case of reduced and bottlenecked populations and they are especially vulnerable to the effects of genetic impoverishment (Pimm *et al.* 1988, Frankham 1995). This vulnerability as well as their phenotypic, genotypic and ecological particularities has promoted their study and conservation (Whittaker & Fernández-Palacios 2007). In addition, their relatively simple, discreet and finite character facilitates the study of evolutionary mechanisms and genetic variability in these populations (Miller *et al.* 2010; Aguilar *et al.* 2004).

Traditionally, studies of population genetics in threatened populations have used presumably neutral genetic markers, such as mtDNA or microsatellites (Frankham *et al.* 2002). However, patterns of neutral genetic variation do not necessarily correlate with those in functionally important genes (e.g. Aguilar *et al.* 2004). Hence, the parallel study of adaptive genetic variation and the mechanisms underlying such variation is recommended, in order to enhance our understanding of the repercussions of decreased genetic diversity in threatened populations (Hedrick 2000; Kohn *et al.* 2006). Genes of the Major Histocompatibility Complex (MHC) are among the best-studied functional genes in wild populations and constitute one of the most important components of the vertebrate immune system. They are involved in the triggering of adaptive immune responses by coding for cell-surface glycoproteins that bind and present foreign peptides

(antigens) to CD4+ and CD8+ T lymphocytes (Klein 1986; Iwasaki & Medzhitov 2010). The recognition of foreign antigens bound to MHC molecules by T lymphocytes initiates a series of immune mechanisms that includes the production of specific antibodies and the destruction of pathogen-infected cells. MHC genes encompass the most polymorphic coding regions of vertebrate genomes (e.g. Robinson *et al.* 2005). This variation is thought to determine the capability of individuals to respond to continuously evolving pathogens and parasites, and consequently, MHC diversity has been traditionally associated with individual fitness and outcome of infection (reviewed by Oliver *et al.* 2009; Radwan *et al.* 2009; Spurgin & Richardson 2010).

Demographic processes, population structure, sexual selection and selective pressures imposed by pathogens are expected to drive the evolution of MHC genes of adaptive significance (Piertney & Oliver 2006; Hedrick 2002). However, empirical studies comparing neutral and adaptive loci have not provided unequivocal results with regard to the relative roles of neutral evolutionary forces and natural selection. In some cases, balancing selection has been invoked to explain the maintenance of high levels of MHC diversity and lower inter-population differentiation in comparison with neutral markers (Aguilar *et al.* 2004; van Oosterhout *et al.* 2006; Mona *et al.* 2008). Other studies, on the contrary, have pointed towards a predominant role for genetic drift in the distribution of MHC variation (e.g. Miller & Lambert 2004a; Mainguy *et al.* 2007, Babik *et al.* 2008, Miller *et al.* 2010). Both simulated and empirical data also suggest that the loss of genetic diversity can occur faster when genetic drift and directional selection occur simultaneously in small and isolated demes (e.g. Ejsmond & Radwan 2009, Alcaide *et al.* 2010). Finally, the role of pathogen diversity and virulence in shaping genetic variation at evolutionarily relevant MHC genes has been stressed by other studies (e.g. Prugnolle *et al.* 2005; Dionne *et al.* 2007; Alcaide *et al.* 2010).

The aim of the present study is to investigate the role of neutral evolutionary forces and selection on the distribution of functional variation in reduced populations. For this purpose we integrate neutral (microsatellites and mtDNA) and adaptive (MHC class II) genetic information in insular and continental populations of the globally threatened Egyptian vulture (*Neophron percnopterus; Accipitridae*). This vulture is one of the few large raptors able to colonize oceanic islands and included insular populations in the Atlantic Ocean and the Mediterranean and Arabic Seas, though many of them are now extinct (Levy 1996; Sarà *et al.* 2009; Gangoso *et al.* 2006). Despite its broad distribution range (Fig. 1), it is estimated that only 30,000 to 40,000 mature individuals survive in the entire world and the species is considered "Endangered" (BirdLife International 2008). In the Western Palearctic, the Iberian Peninsula (Iberia) holds the bulk of the European population and the two main insular demes are located in Menorca (Balearic Islands) and Fuerteventura (Canary Islands) (Fig. 1). All of these populations are sharply declining (BirdLife International 2008; Donázar *et al.* 2001; De Pablo 2002).

We specifically assessed which among the following hypotheses is better supported by our empirical data: i) patterns of MHC variation cannot be discerned from those occurring at neutral markers (i.e. equivalent extent of genetic divergence and similar loss of variability at neutral and adaptive loci), thus indicating a predominant role of neutral forces over selection; ii) balancing selection counteracts genetic drift and therefore both population differentiation and loss of genetic diversity at MHC loci has been minimised and iii) population divergence at the MHC exceeds that observed at neutral markers and this finding supports diversifying selection.

Methods

Sampling and nucleic acid extraction

The present study was carried out with samples from the main continental (Iberia) and the two relic insular populations (Menorca in the Balearics and Fuerteventura in the Canary Islands) in the Western Palearctic (Fig. 1). Blood samples were obtained from vultures captured at their nests or with cannon nets at supplementary feeding areas between 1995 and 2006 in Iberia from northern (Navarre; n=40 and Aragon; n=19), central (Segovia; n=15) and southern (Cadiz; n=22) locations. Samples were taken between 1998 and 2009 in Fuerteventura (n=236) and between 1998 and 2002 in Menorca (n=36). DNA extracts were obtained following Sambrook *et al.* (1989).

Genotyping of microsatellite and mtDNA markers

Estimates of neutral genetic variation were based on the survey of five non-specific (Bv6, 9, 13, 14 and 20; Gautschi *et al.* 2000) and 17 species-specific microsatellite loci (Np38, 39, 51, 78, 93, 140, 141, 155, 163, 166, 229, 238, 244, 249, 257, 259, 296; Agudo *et al.* 2008) accomplished in a recent study published by the authors (Agudo *et al.* 2011). We used GENALEX version 6 (Peakall & Smouse 2006) to calculate parameters of genetic variability and differences between populations were tested using Wilcoxon sign-rank tests. In addition, we sequenced 403 bp of the mitochondrial control region (domain I) using primers THR-F and FBox-R and following the conditions described in Roques *et al.* (2004). PCR amplicons were directly sequenced according to the BigDye 1.1 technology (Applied Biosystems, Carlsbad, California). Labelled fragments were analysed using an ABI 3130xl automated sequencer (Applied Biosystems, Carlsbad, California). DNA sequences were edited using BioEdit (Hall 1999) and

polymorphism statistics were generated with DNAsp 5.0 (Librado & Rozas 2009). Sample sizes used for each kind of marker are given in Table 1.

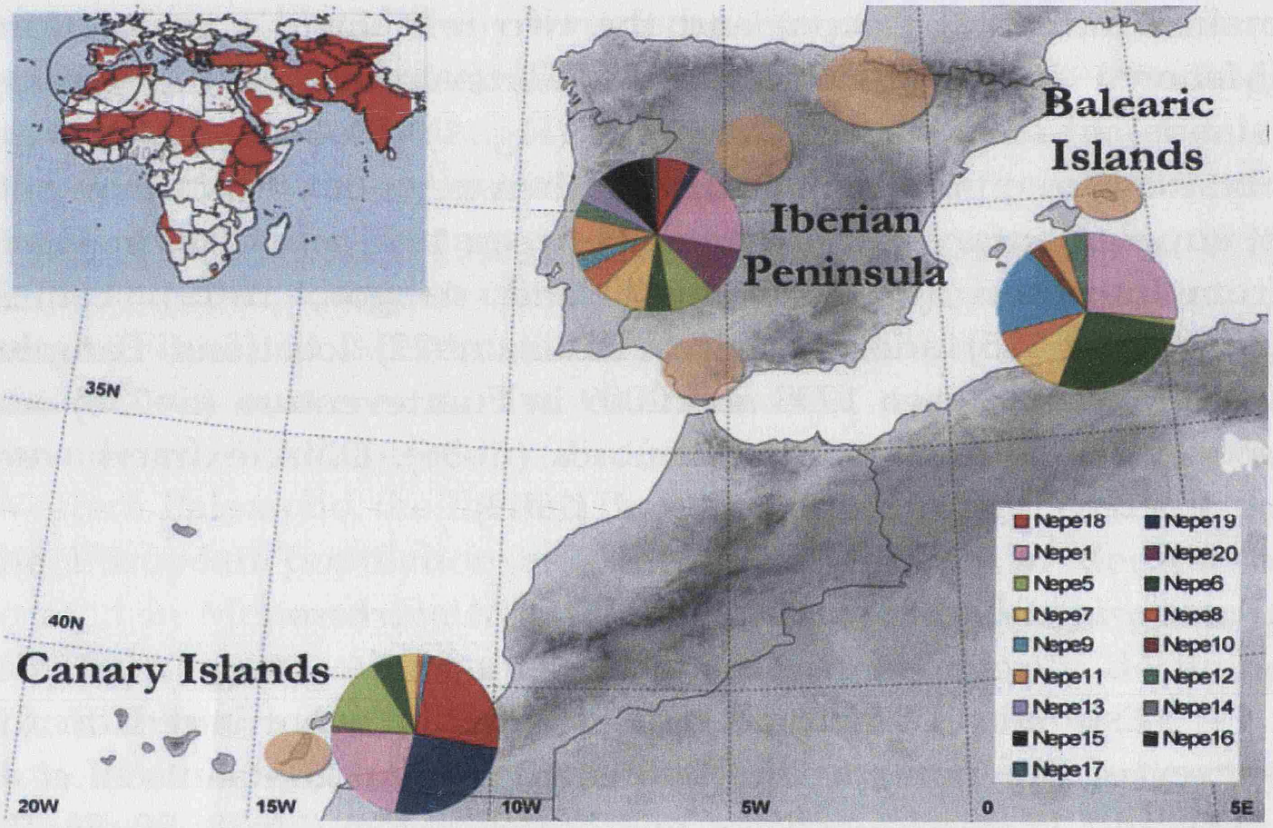


Figure 1 Global distribution (upper left corner) and geographic location of sampled areas (orange circles) in Iberia and the two insular populations of the Egyptian vulture (Fuerteventura in the Canary Islands and Menorca in the Balearic Islands) investigated in this study. Pie charts represent the frequency distribution of 17 MHC class II alleles. Each allele is represented by a different colour.

MHC genotyping and molecular cloning

The second exon of MHC class II B genes was PCR amplified using the primers Acc2FC and Acc2RC according to Alcaide *et al.* (2007). We performed capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) analyses to

resolve MHC class II genotypes (e.g. Bryja *et al.* 2005; Alcaide *et al.* 2010). Fluorescently labelled PCR products were diluted 1:10 and denatured using Hi-Di formamide, and heated at 95°C for 3 min. SSCP analyses were run on an ABI 3130xl automated sequencer (Applied Biosystems) using a polymeric matrix composed of a 5% CAP non-denaturing polymer (Applied Biosystems, Carlsbad, California), 10% glycerol and 1x Genetic Analyzer running buffer. Samples were run at 33°C with 0.7 µl of GeneScan 500-LIZ size standard, using the FragmentAnalysis36_POP4 run module. SSCP electropherograms were analysed using GenMapper 3.7 (Applied Biosystems, Carlsbad, California).

Eight birds from each population suspected to host different alleles were re-amplified using non-labelled primers and cloned into bacterial plasmids using the PGEM-T easy vector system II (Promega). We randomly sequenced 8-12 positive clones per individual to isolate MHC class II β alleles. Samples representing all unique SSCP profiles were directly sequenced as well. MHC allele sequences were assigned to particular SSCP peaks through the comparison of those SSCP electropherograms that apparently shared alleles. For every SSCP genotype, we carefully examined whether the overlap of DNA sequences of the inferred alleles matched the nature of the polymorphic sites detected across sequencing chromatograms. Allele sequences and direct sequencing chromatograms were edited in BioEdit (Hall 1999). Polymorphism statistics were generated with DNAsp ver 5.0 (Librado & Rozas 2009). The phylogenetic relationships among MHC alleles were visualized through Neighbor-net networks built with SplitsTree 4.0 (Huson & Bryant 2006).

Finally, we investigated the MHC allele segregation patterns from parents to offspring in 33 families of Egyptian vultures (n=236 individuals) sampled from the Canary Islands. Kinship relations were reconstructed according to field observations and molecular data (Agudo *et al.* 2011). The observation of allele inheritance allowed us to: i) obtain

information regarding the overall number of loci co-amplified, ii) test whether or not they are linked, and iii) evaluate the possibility of assigning alleles to particular loci.

Tests of selection

Frequencies of non-synonymous (d_N) and synonymous (d_S) nucleotide substitutions were calculated using the software MEGA 4.0 (Kumar *et al.* 2008). An excess of d_N over d_S (d_N / d_S = ω >1) provides evidence of historical positive selection. Tests were run independently for those codons supposedly involved and not involved in antigen recognition, according to the crystallographic structure of the human MHC class II molecule (Brown *et al.* 1993). Deviations from neutral expectations of molecular evolution were also tested through a Tajima's D test (Tajima 1989) using DNAsp ver 5.0 (Librado & Rozas 2005) with a sliding window of 25 nucleotides and a step size of 5 nucleotides.

Positive selection at every codon was also tested using the HyPhy package (www.datamonkey.org, Pond *et al.* 2005). We ran the GARD module (Kosakovsky Pond *et al.* 2006) to subdivide our alignment into different partitions on the basis of significant evidence of recombination breakpoints, as recombination is known to seriously overestimate positive selection (Richman *et al.* 2003; Anisimova *et al.* 2003). We selected the Random Effects Likelihood (REL) model to infer site-by-site positive selection (Kosakovsky Pond & Frost 2005) considering only Bayes factors larger than 100 (i.e. those supporting decisive evidence of positive selection) obtained independently with different nucleotide substitution models.

Since there was not clear clustering of alleles according to locus and there were evidences that the two gene duplicates may be sharing the same set of alleles (see discussion below), we included all exon 2 sequences in the analysis of positive selection.

Estimates of population differentiation

First, we used the nucleotide sequence-based estimator of population genetic differentiation K_{ST} (Hudson *et al.* 1992) to investigate genetic divergence at MHC and mitochondrial loci (p-values were calculated by running 1,000 iteration permutation tests) using DNAsp 5.0 (Librado & Rozas 2009). Second, we calculated pairwise F_{ST} values at MHC loci (see below) and at mitochondrial DNA and microsatellite loci using DNAsp 5.0 and FSTAT 2.9.3 (Goudet 1995) respectively. For the microsatellites, 95% confidence intervals were assessed by bootstrapping over loci. For the calculation of K_{ST} and F_{ST} for the MHC genes, we first calculated the allele frequencies by considering the sequence of a particular allele as many times as the inferred number of copies of that allele in a given individual (including the two gene copies). F_{ST} was estimated using Arlequin 3.1 (Excoffier *et al.* 2005). After that and based on the presence of clear groups of linked alleles within the islands, we also calculated the F_{ST}-value at MHC genes between the two insular populations by using the frequencies of the different pairs of alleles in LD (rather than individual alleles) using Genetix 4.04 (Belkhir *et al.* 2004) (see results). MHC F_{ST}-values that did not overlap within the 95% confidence intervals of those based on microsatellites were considered significantly different (Weir 1996). All individuals used for population differentiation analyses based on both neutral and adaptive loci were presumably unrelated. Given that our main goal was to analyze the differences between the continental and the insular populations and since, we did not find evidence of partial structure within Iberia, analyses of population differentiation were performed considering all continental individuals as a single cluster.

Results

Genetic variation at microsatellite, mitochondrial and MHC loci

The genotyping of 22 microsatellite markers reported higher levels of expected heterozygosity (H_E) and allele richness (AR) in the mainland (0.57 and 3.06) than in the two insular demes (Fuerteventura: H_E =0.44, AR =2.44 and Menorca: H_E =0.53, AR=2.59; Wilcoxon sign-rank tests between Iberia and: a) Fuerteventura H_E: Z=2.46 / p=0.01, AR: Z=2.13 / p=0.03 and b) Menorca H_E: Z=1.86 / p=0.06; AR: Z=2.03 / p=0.04) (data taken from Agudo *et al.* 2011). The analysis of 403 bp of the mitochondrial control region revealed a ratio of 0.80 haplotypes per individual in Iberia, 0.57 in Menorca and 0.38 in Fuerteventura (Table 1).

Overall, we discriminated among 17 MHC class II alleles in the Egyptian vulture (Fig. 2, waiting for GenBank accession numbers). All alleles were independently found in at least two individuals except for two alleles (Nepe14 and Nepe17) that were only detected in two continental birds. Polymorphism statistics across the MHC class II data set revealed 37 segregating (variable) sites corresponding to 40 mutations, a nucleotide diversity among alleles (π per site = 0.042) and 11.35 nucleotide differences, on average, between alleles (Table 1). All alleles differed by at least one non-synonymous substitution, which suggests that they might also differ in their antigen binding properties. The individual survey of MHC variability reported 2, 3 or 4 different alleles per individual which is in agreement with the simultaneous amplification of two gene duplicates in the Egyptian vulture.

The Iberian population exhibited the highest degree of MHC variability. We found 17 alleles and 28 MHC class II genotypes across the 30 individuals screened. Eleven alleles were observed in the three studied Iberian subpopulations and the most

abundant allele (Nepe1) was so for these three sampled demes independently. Only low-frequent alleles (N=6) were not found in the three demes, suggesting a random effect linked to the small sample sizes (N=10). In agreement with the relatively weak genetic differentiation observed at nuclear markers among these subpopulations (Fst<0.03, Agudo *et al.* 2011), our data do not suggest a strong genetic structuring at the MHC either. As a result, we herein consider the Iberian Peninsula as a whole.

Only 10 class II alleles and 9 genotypes were found in Fuerteventura (n=236), and five of them (G1-G5, Fig. 1 and S1) accounted for near the 90% of the genotypic variation in this population (Table 2). Likewise, only 9 alleles and 11 class II genotypes were inferred in Menorca (n=36) (Fig.1 and S2). Mean number of MHC alleles per individual was also higher in the continent (3.37) than in the islands (2.93 for Fuerteventura and 2.96 for Menorca, Table 1).

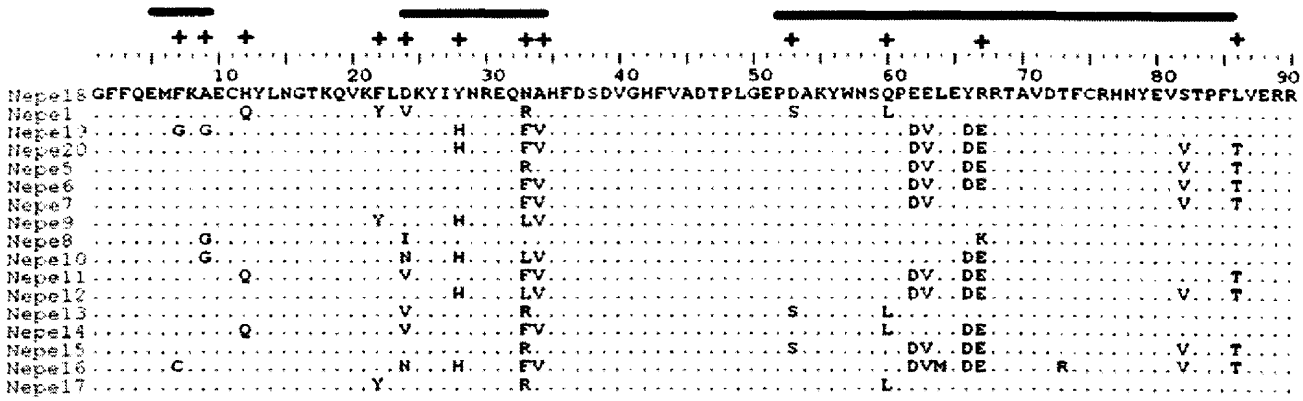


Figure 2 Putative amino acid sequences of 17 MHC class II alleles (β chain, exon 2) in the Egyptian vulture. Dots indicate a match to the top sequence and crosses pinpoint codons exhibiting strong evidence of positive selection (see also Fig. 5). Black bars cover the three major regions comprising the antigen-binding region of the human MHC class II molecules (Brown *et al.* 1993).

Table 1 Polymorphism statistics at a mitochondrial control region locus (403 bp of subdomain I), 22 microsatellites and two MHC class II β duplications in one mainland (Iberia = IBE) and two insular populations (Fuerteventura = FV; Menorca = MEN) of the Egyptian vulture. Microsatellite diversity is measured in terms of allele richness (*AR*) and average expected heterozygosity (H_E). Mean nucleotide diversity per site (π) and the average number of nucleotide differences among alleles (k) are indicated for mitochondrial and MHC loci. For the later, the overall number of alleles (*Na*), the total number of different genotypes (*Ng*), the mean number of alleles per individual (*Na/ind*) and the total number of segregating sites (*S*) in the alignment are also shown.

	Populations		
	FV	**MEN**	**IBE**
MtDNA control Region (N)	13	7	40
No. of haplotypes	5	4	32
π	0.007	0.01	0.024
k	2.73	4.17	9.65
MHC (N)	30	24	30
Na	10	9	17
Ng	9	11	28
Na/Ind	2.93	2.95	3.36
S	28	25	30
π	0.047	0.043	0.044
k	11.98	11.69	11.99
Microsatellite (N)	45	36	96
AR	2.44	2.59	3.06
H_E	0.44	0.53	0.57

Table 2 Allele composition and frequencies of the different genotypes observed in 236 Egyptian vultures from Fuerteventura). Genotypes composed of 2 alleles were assumed to have two copies of each allele (see also Fig. S1 and Fig. S2 for inferred genotypes in Menorca).

Genotype	Allele1	Allele2	Allele3	Allele4	Frequencies
G1	Nepe18	Nepe19			0.259
G2	Nepe1	Nepe5			0.174
G3	Nepe18	Nepe19	Nepe1	Nepe5	0.436
G4	Nepe18	Nepe19	Nepe1	Nepe6	0.017
G5	Nepe1	Nepe5	Nepe6		0.009
G6	Nepe1	Nepe7	Nepe5		0.076
G7	Nepe1	Nepe7			0.004
G8	Nepe1	Nepe20	Nepe7	Nepe11	0.004
G9	Nepe1	Nepe5	Nepe8	Nepe9	0.017

The absence of specific allele combinations in Fuerteventura, not only in the offspring but also across the entire population, indicated that the two MHC gene duplicates are necessarily linked (Table 3, Fig. 3A). Furthermore, the observed allele segregation patterns suggest the existence of different alleles within the linkage groups (Fig 3B, 3C and 3D). This result rejected the possibility that the two duplicates could hold identical exon 2 alleles mapped to the same chromosome, as previously shown in pheasants (Wittzell *et al.* 1999). We identified 6 different linkage groups in Fuerteventura (Table 2, Fig. S1). Genotypes composed of 2 alleles were assumed to have two copies of each allele. In the case of genotypes with 3 alleles, duplicated alleles were deduced by observing the linkage groups involved in that particular genotype (e.g. G5 and G6, Fig. S1).

In Menorca, two genotypes were composed of only two alleles: Nepe3 + Nepe6 and Nepe9 + Nepe6, respectively (Fig. S2) and were considered as linkage groups in this population. We also observed three additional linkage groups already reported in

Fuerteventura (Nepe8-9, Nepe3-7 and Nepe3-5). Other linkage groups occurred at very low frequencies (Fig. S2).

Averaged value of genetic divergence was higher between linked alleles (9.16±3.25 amino acid differences), than across random allele associations in Fuerteventura, even after discarding self-allele comparisons (7.6±3.26). Differences were even more remarkable when considering the four linkage groups that constituted 90% of the genotypes (G1-G7; 11.±1.63, see Figure S1). A similar trend was also detected in Menorca, where the two main linkage groups (Nepe3+6 and Nepe9+6) exhibit 13 and 9 different amino acid substitutions between alleles, respectively (Fig. S2).

In the case of Iberia, the comparably larger number of genotypes prevented us from inferring putative linkage groups with confidence. When calculating individual allele frequencies, we kept on considering that those genotypes constituted by two alleles should include two copies of each particular allele. Only 6 out of the 30 continental birds displayed three alleles. Following the experience collected from examples such as G5 and G6 genotypes in Fuerteventura (Fig. S1), we assumed that the allele emitting more fluorescence was occurring in two copies.

Historical selection at the MHC

Non-synonymous substitutions were significantly more frequent than synonymous substitutions in those codons presumably comprising the antigen-binding sites (d_N = 0.145±0.055; d_S = 0.043±0.031, Z-test statistic p=0.046), but not outside these regions (d_N = 0.028±0.010; d_S = 0.011±0.008, Z-test statistic p=0.23). A sliding-window Tajima's D test revealed three coding regions with significant and positive Tajima's D values (Fig. 4). This indicates an excess of high-frequency segregating (variable) sites and supports the idea of balancing selection.

GARD analyses reported a recombination breakpoint in the nucleotide position 158 of exon 2 (p<0.001). The codon-based

maximum likelihood REL method revealed evidence of positive selection (all Bayes factors >100) in 12 amino acid positions and negative selection in two codons of the alignment (Fig. 5).

Table 3 Crosses between paternal and maternal MHC genotypes (G1-G9, see Fig. S1) in 33 families of Canarian Egyptian vultures. Number of times that a particular crossing was observed is indicated (N) and the number of offspring showing a particular genotype is shown in brackets.

	N	Offspring Genotypes
G1 x G1	1	G1 (2)
G1 x G2	1	G3 (6)
G1 x G3	7	G1 (4); G3 (5)
G1 x G4	2	G1 (2); G4 (2)
G2 x G3	6	G2 (5); G3 (10)
G2 x G6	2	G2 (3); G6 (5)
G2 x G9	1	G9 (3)
G3 x G3	8	G1 (8); G2 (1); G3 (11)
G3 x G6	4	G2 (5); G3 (4); G6 (3)
G3 x G7	1	G6 (1)

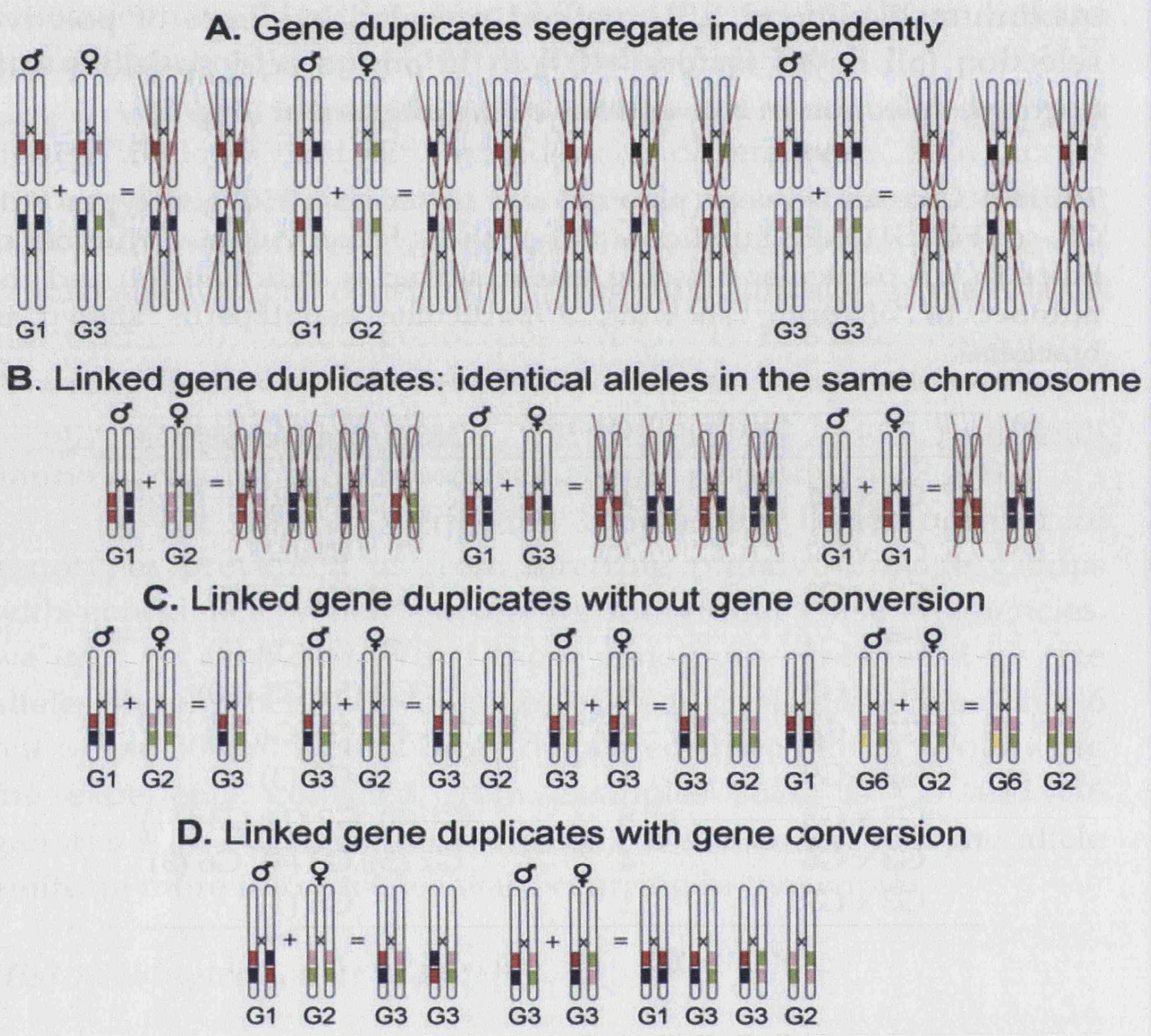


Figure 3 Hypothetic allele configurations across two MHC class II β gene duplications in the Egyptian vulture. Parallel bars represent homologous chromosomes and colours represent the different alleles observed in the Canarian population (red=Nepe18; dark blue=Nepe19; pink=Nepe1; violet=Nepe20; light green=Nepe5; dark green=Nepe6; yellow=Nepe7; orange=Nepe8; cyan=Nepe9; sepia=Nepe11). Some of the expected genotypes (indicated by red crosses) were found neither in the offspring nor in the entire population. **A)** Expected segregation of alleles in the case of two MHC genes duplicates segregating independently. **B)** Linked genes but identical alleles mapping to the same chromosome. **C)** Linked genes including different alleles within each linkage group for two evolutionarily independent genes. **D)** Linked genes in the same conformation but assuming gene conversion (i.e. both gene duplicates can share the same alleles). The investigation of allele segregation patterns rejected both A and B hypotheses.

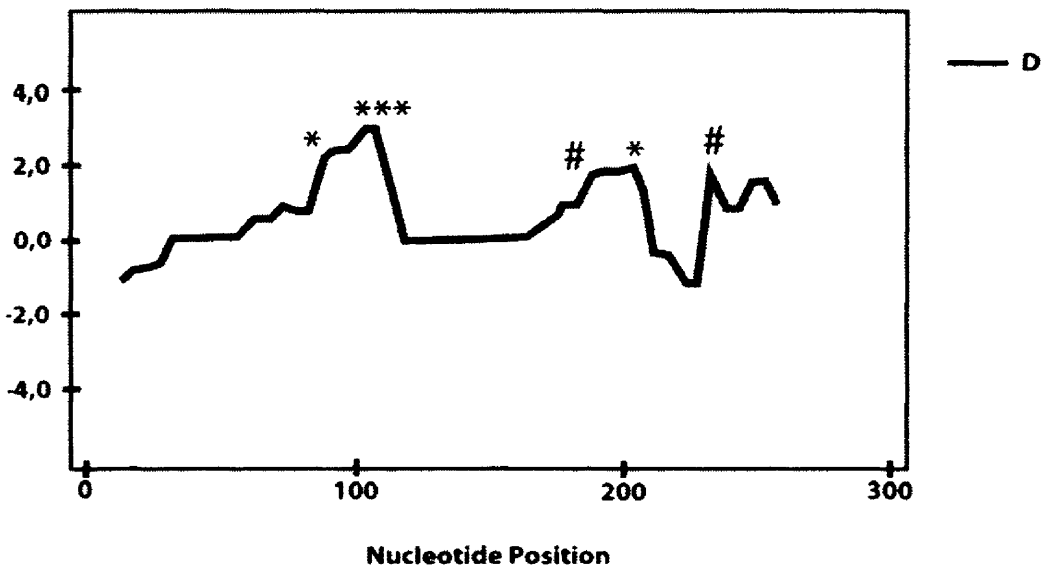


Figure 4 Sliding window estimation of Tajima's D value along the second exon of MHC class II β genes in the Egyptian vulture. Regions displaying statistically significant and positive Tajima's D value are indicated (***p<0.001, *p<0.01, #p<0.1).

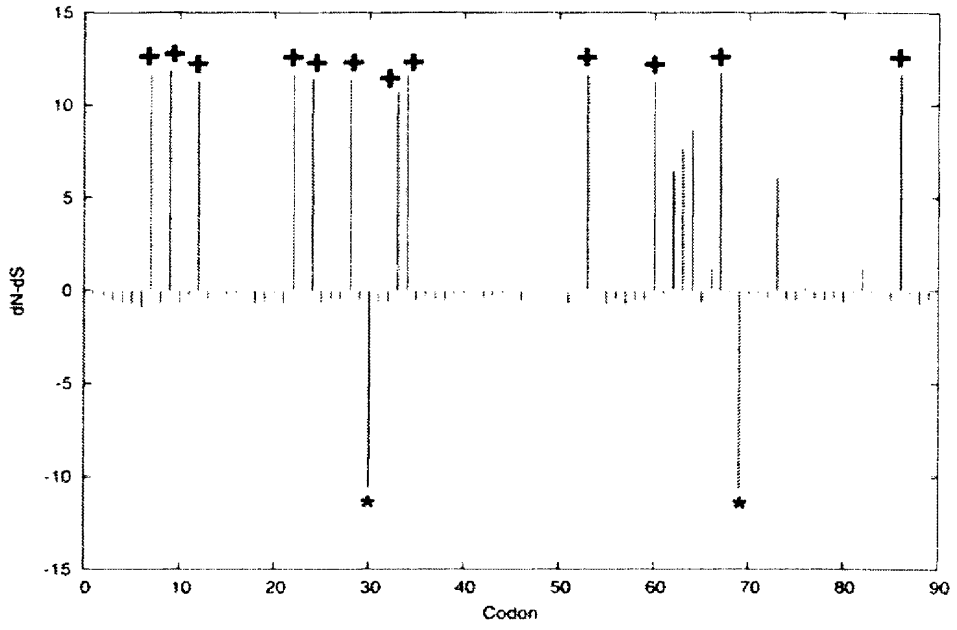


Figure 5 Balance between non-synonymous (d_N) and synonymous (d_S) substitution rates for every single codon across 17 MHC class II alleles in the Egyptian vulture. Crosses indicate decisive evidence of positive selection (Bayes factors > 100) and asterisks indicate statistical evidence for negative selection.

Table 4 Estimates of population differentiation at mitochondrial, microsatellite and MHC loci between a mainland (Iberia) and two insular populations (Menorca and Fuerteventura) of the Egyptian vulture. All F_{ST} and K_{ST} values are statistically significant ($p < 0.001$) (95% confidence intervals for microsatellite loci are provided). Pairwise F_{ST} value between the two islands and base on linked allele frequencies are indicated between square brackets.

F_{ST} mtDNA / MHC μsats	**Menorca**	**Fuerteventura [based on linkage groups]**
Iberia	0.172 / 0.056 0.077 (0.051-0.103)	0.290 / 0.058 0.092 (0.045-0.146)
Menorca [based on linkage groups]		0.585 / 0.118 0.138 (0.092-0.179) [0.272]
K_{ST} mtDNA / MHC	**Menorca**	**Fuerteventura**
Iberia	0.042 / 0.013	0.115 / 0.027
Menorca		0.417 / 0.034

Patterns of population differentiation

Estimates of population differentiation were in agreement with a pattern of isolation by distance with the highest differentiation between the two insular populations regardless of the type of marker analysed. The distribution of the pairwise differences in the MHC allele frequencies between the mainland and insular populations was similar to that observed at neutral loci (Fig. 6). Pairwise F_{ST} values at MHC loci based on the inferred allele frequencies fell within the 95% confidence intervals reported for microsatellites (Table 4). Rates of allele fixation at the MHC were about four times slower than those observed for

mitochondrial haplotypes, which is in agreement with the four times higher effective population size of nuclear MHC loci. However, when using the frequencies of linkage groups rather than individual allele frequencies to calculate the MHC F_{ST} value between the islands, we found that it was significantly larger (F_{ST} = 0.272) than that at microsatellite loci (F_{ST} = 0.138; Table 4). Finally, nucleotide-based estimators of population differentiation suggested a comparably slower fixation of variable sites at MHC loci than at mitochondrial sequences. The fact that mitochondrial K_{ST} values between the two insular populations exceeded by one order of magnitude those reported for the MHC is exemplary in this regard (Table 4).

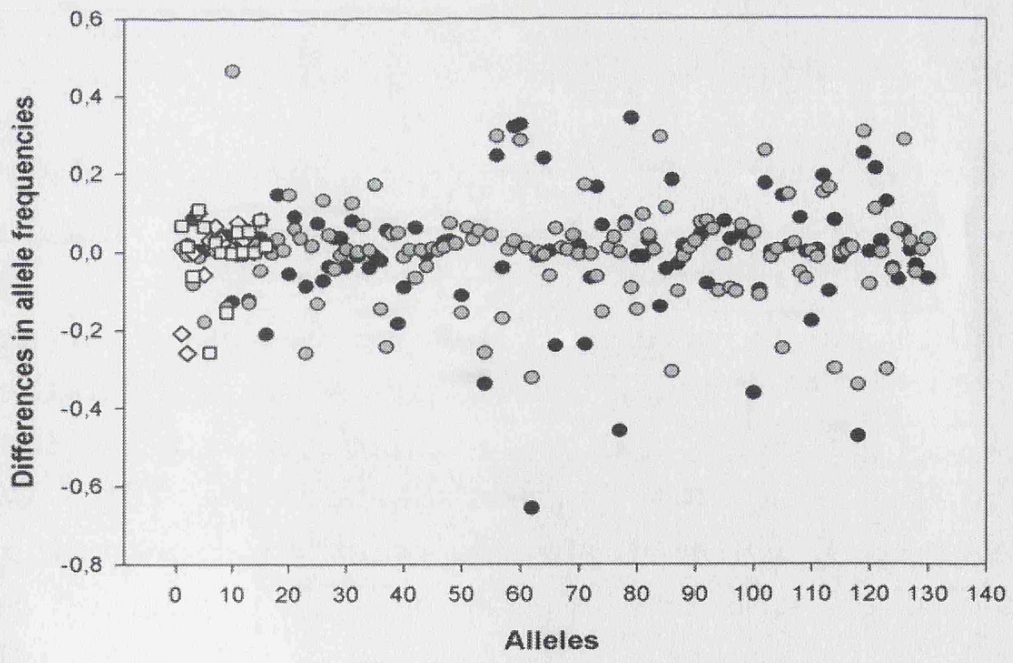


Figure 6 Pair wise differences in allele frequencies. Every spot symbolizes one allele: black spots indicate the difference in allele frequencies of 130 alleles (corresponding to 22 microsatellite loci) between Iberia and Fuerteventura. Grey spots correspond to the differences between Iberia and Menorca. Empty diamonds represent frequency differences of 17 MHC alleles between Iberia and Fuerteventura and empty squares represent differences in MHC allele frequencies between Iberia and Menorca.

Discussion

This study integrates neutral and MHC variation to disentangle the partial role of neutral evolutionary forces and selection on the distribution of adaptive variation in bottlenecked and geographically isolated populations. Overall, our findings suggest a major role of genetic drift in shaping the spectrum and frequencies of MHC alleles across island populations of Egyptian vultures. However, the most intriguing result of the present study is the putative co-evolution of gene duplicates in the Egyptian vulture. We hypothesize that this evolutionary mechanism may counteract allele fixation, maximize antigen recognition capabilities and promote the co-inheritance of selectively advantageous alleles in genetically depauperate populations.

MHC variation and population differentiation

Our results indicate the existence of two MHC class II gene duplicates in the Egyptian vulture, as previously suggested by Alcaide *et al.* (2007) in this and other *Accipitridae* species. Compared to other scavengers, the MHC class II of Egyptian vultures seems less variable than that of vultures from the genus *Gyps* (Alcaide *et al.* 2007) but more variable than the MHC class II of Andean Condors (*Vultur gryphus*) (Alcaide *et al.* 2010). We must recall however, that the 30 studied continental individuals may not hold all the alleles presented in as large population as Iberia and therefore, we may be underestimating the genetic diversity present in the species. Furthermore, SSCP and direct sequencing analyses might have also failed in discriminating among sets of closely related alleles. Although we did not perform gene expression analyses, several lines of evidence sustain the functionality of the loci we investigated. First, we did not detect stop codons or frame shift mutations in any coding region. Second, we found significant support for positive selection acting

on functionally relevant codons (Fig. 2) and an excess of high-frequency segregating (variable) sites (Fig. 4) supports historical balancing selection acting upon antigen-binding sites.

As expected, we observed lower levels of genetic diversity in insular demes than in their continental counterpart (Yuhki & O'Brien 1990; Hedrick *et al.* 2000). Furthermore, comparative analysis of population structure between presumably neutral and MHC loci does not suggest the occurrence of balancing selection counteracting genetic drift (e.g. Aguilar *et al.* 2004, van Oosterhout *et al.* 2006) or local selection accelerating allele fixation (e.g. Esjmond & Radwan 2009, Alcaide 2010) in the studied insular populations, when considering the distribution of individual allele frequencies alone. Therefore, our results remain in the same line as some previous studies that underscored a prominent role for neutral evolution over selection in small, bottlenecked and isolated populations (Miller *et al.* 2004, Manguy *et al.* 2007, Babik *et al.* 2008, Miller *et al.* 2010).

However, when estimating the MHC F_{ST} value between the insular populations based on the frequencies of linkage groups, we observed higher differentiation than expected under neutrality. Even though this result may suggest the action of diversifying selection between the insular populations (Bernatchez & Landry 2003), we cannot rule out the potential contribution of population bottlenecks in the increase of LD between different alleles (McVean 2002). Nevertheless, these islands are located thousands of kilometres apart and are close to different continents and consequently, spatial variations in the pathogen communities must be expected (e.g. de Bellocq *et al.* 2002; Blanco *et al.* 2007; Alcaide *et al.* 2010). We therefore speculate that the strong differentiation in the composition of the dissimilar linkage groups might be the result, in part, of spatial variations in pathogen-mediated selective regimes (Prugnolle *et al.* 2005; Dionne *et al.* 2007; Alcaide *et al.* 2010).

We must also recall though that the comparison between MHC and mitochondrial and microsatellite markers is probably not the most appropriate. High rates of back-mutation and homoplasy are expected to underestimate population differentiation when using microsatellites. Selective sweeps, on the other hand, may increase genetic divergence at mitochondrial DNA sequences (Galtier *et al.* 2009). Therefore, the use of anonymous loci could be more enlightening in these kinds of studies (Lee & Edwards 2008).

Putative co-evolution of MHC gene duplicates

The observed pattern of MHC allele distribution could be the result of divergent alleles displayed by evolutionarily independent genes. However, the finding of linkage groups (e.g.Nepe8+Nepe9; Figs. S1, S2) comprised by very similar alleles on islands (although at the lowest frequencies) and the impossibility of assigning alleles to loci (Fig. S4), reinforce concerted evolution, as previously reported within the *Accipitridae* family (Alcaide *et al.* 2007). In fact, cloning of MHC genomic fragments has revealed an identical intron 2 and exon 3 sequences across the two gene duplicates in Egyptian vultures (GenBank acc. numbers EF370964.1 and JF313939, authors' unpublished data). Furthermore, it is well documented that allele shuffling between gene duplicates can be facilitated by their physical proximity in the genome (Ezawa *et al.* 2006). The sharing of the same battery of alleles between two MHC gene duplicates has already been described in the pheasant (Witzell *et al.* 1999) and found in other species within the *Accipitridae* family such as the Black Kite (*Milvus migrans*) (L. López and M. Alcaide unpublished data). Nevertheless, elucidating whether different alleles have become fixed at different loci in the islands or, if on the contrary, genes are sharing the same battery of alleles require future research. In fact, both scenarios may be consistent with the observed patterns of

genetic inheritance in the Canarian Egyptian vultures (see Fig. S3C and S3D). The design of locus-specific primers or the sequencing of the 5'-UTR region (Worley *et al.* 2008, Miller & Lambert 2004b) could provide conclusive evidence in this respect.

Even though we are aware of the putative influence of population bottlenecks on the observed LD patterns (McVean 2002), we hypothesize that our findings may be in agreement with the co-evolution of gene duplications into MHC haplotypes, as previously described by Kaufman (1999) in the domestic chicken *Gallus gallus*. This theory suggests that selectively advantageous allele combinations can be kept together in the genome over long periods of time. Certainly, positive selection of linkage groups containing divergent alleles guarantees the possession of at least two alleles with contrasting antigen binding properties. In this regard, gene duplication would allow not only acquiring new functions (Dittman & Liberles 2010), but also counteracting the deleterious effects of genetic drift and promoting the co-inheritance of selectively advantageous alleles. This evolutionary mechanism would be especially beneficial in bottlenecked and decimated island populations.

Implications in conservation

Even though we herein speculate about the importance of gene co-evolution to maximize MHC variation in island populations, our results highlight a prominent role of genetic drift in the distribution of adaptive variation. As a result, insular and bottlenecked populations present significantly less MHC variability than their continental counterparts. This limitation might compromise their capabilities to confront novel infectious diseases (but see Acevedo-Whitehouse & Cunningham 2006; Radwan *et al.* 2009). In fact, previous research has already shown that island Egyptian vultures present lower immune response capabilities and higher susceptibility to infection than their

continental equivalents (Gangoso *et al.* 2009). Scavenger raptors are particularly at risk (Blanco *et al.* 2007, Lemus & Blanco 2009a, Lemus *et al.* 2008). An alarming increase in pathogenic burdens in both continental and insular populations of these species has recently been described (Blanco *et al.* 2007, Lemus & Blanco 2009a, Lemus *et al.* 2008). This increase has been related to the sanitary regulations associated with the bovine spongiform encephalopathy (BSE) crisis (Tella 2001), the consequent congregation of vultures around carcasses of stabled animals and the massive ingestion of veterinary-prescribed drugs used in the raising of stabled animals (Lemus & Blanco 2009b, Blanco *et al.* 2009). Therefore, this alarming increase in pathogen exposure may have important consequences on the survival of insular populations with lower adaptive flexibility against invading pathogens over the long term.

Supplementary Material

Figure S1

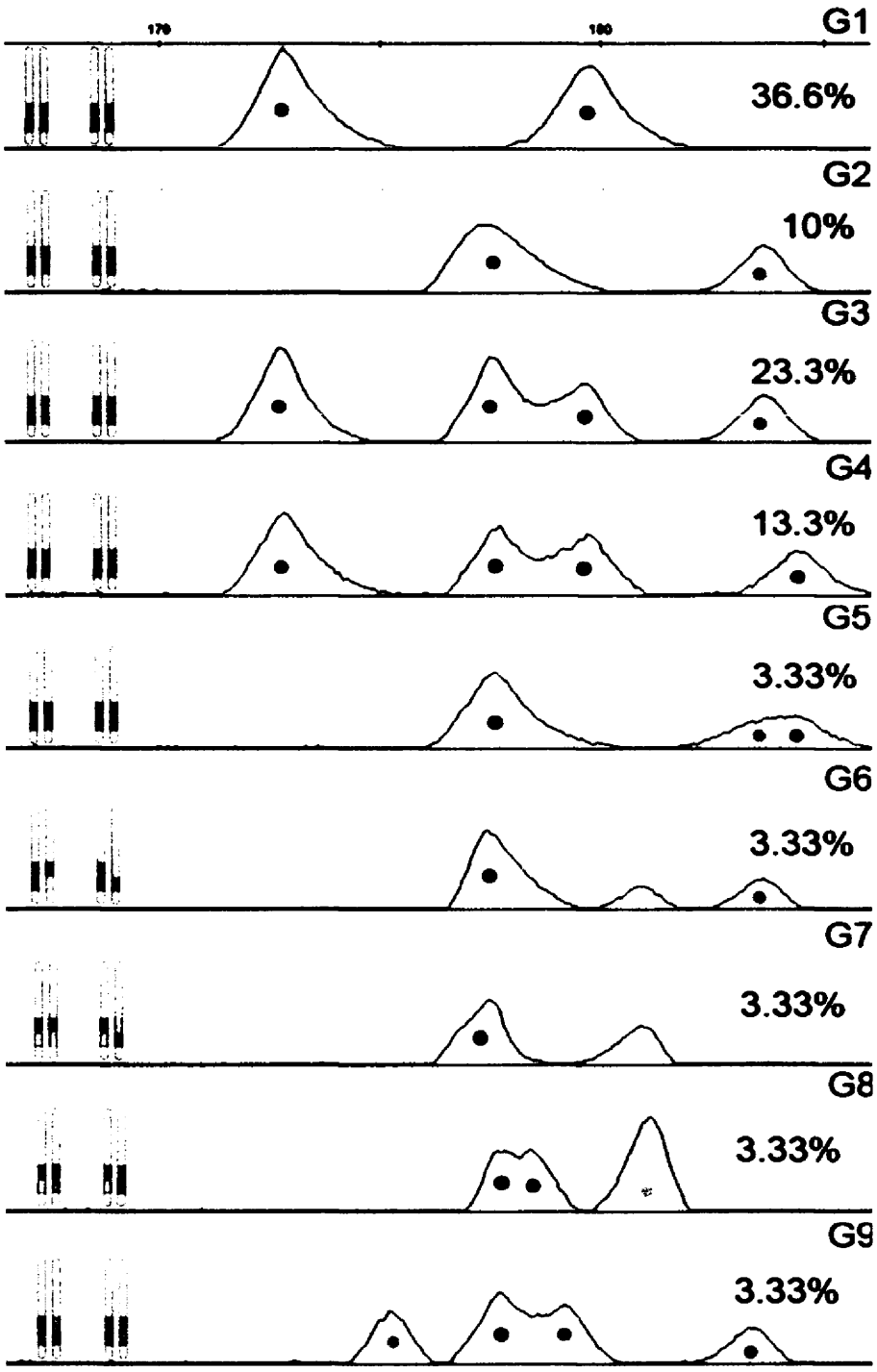


Figure S1. SSCP electropherograms of the 9 MHC class II genotypes identified in 30 non-related Canarian Egyptian vultures. Hypothetical combinations of alelles between the two gene duplications are displayed: left, without assuming gene conversion, and right, assuming gene conversion (see Fig. 3 for details). Schemes represent homologous paternal and maternal chromosomes in G0 phase. Colour dots represent different alleles (red=Nepe18; dark blue=Nepe19; pink=Nepe1; violet=Nepe20; light green=Nepe5; dark green=Nepe6; yellow=Nepe7; orange=Nepe8; cyan=Nepe9; sepia=Nepe11). The relative frequencies of each genotype are also indicated. Only data from the single stranded DNA molecules labelled with the 6-FAM dye is shown.

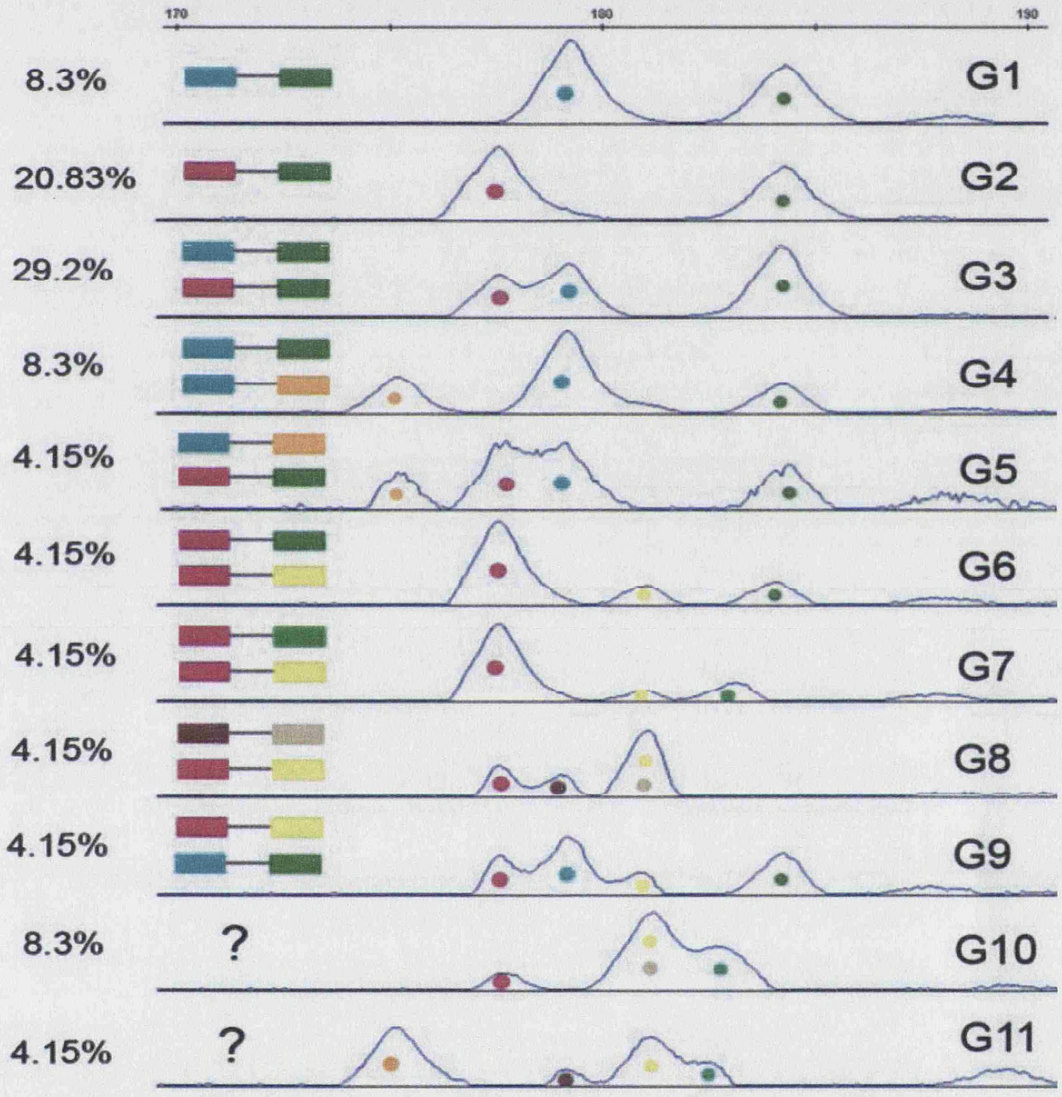


Figure S2. SSCP electropherograms of the 10 MHC class II genotypes identified in the Menorca population of the Egyptian vulture. Linkage groups between the two gene duplicates are suggested. Colour dots represent different alleles (pink=Nepe1; light green=Nepe5; dark green=Nepe6; yellow=Nepe7; orange=Nepe8; cyan=Nepe9; brown= Nepe10; sepia=Nepe11; bright green=Nepe12). The relative frequencies of each genotype are also provided. Only data from the single stranded DNA molecules labelled with the 6-FAM dye is shown.

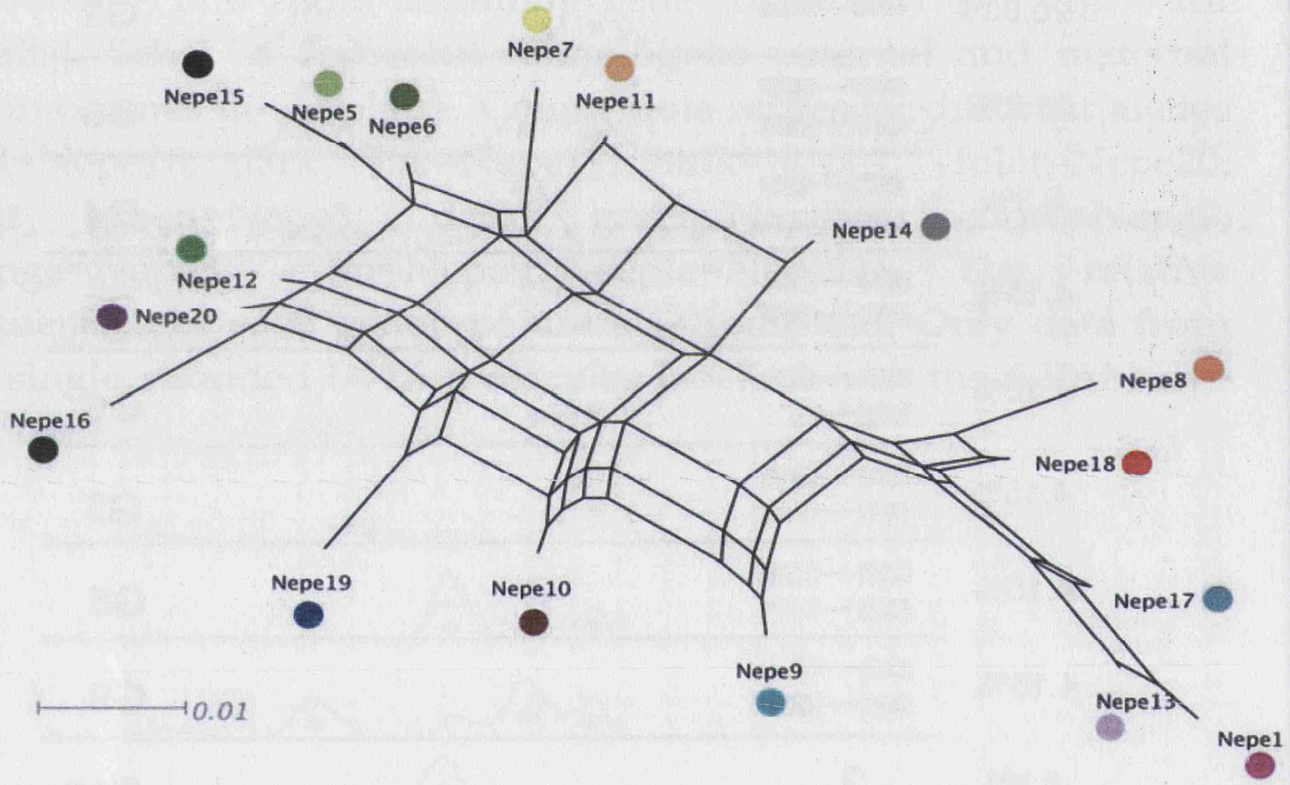
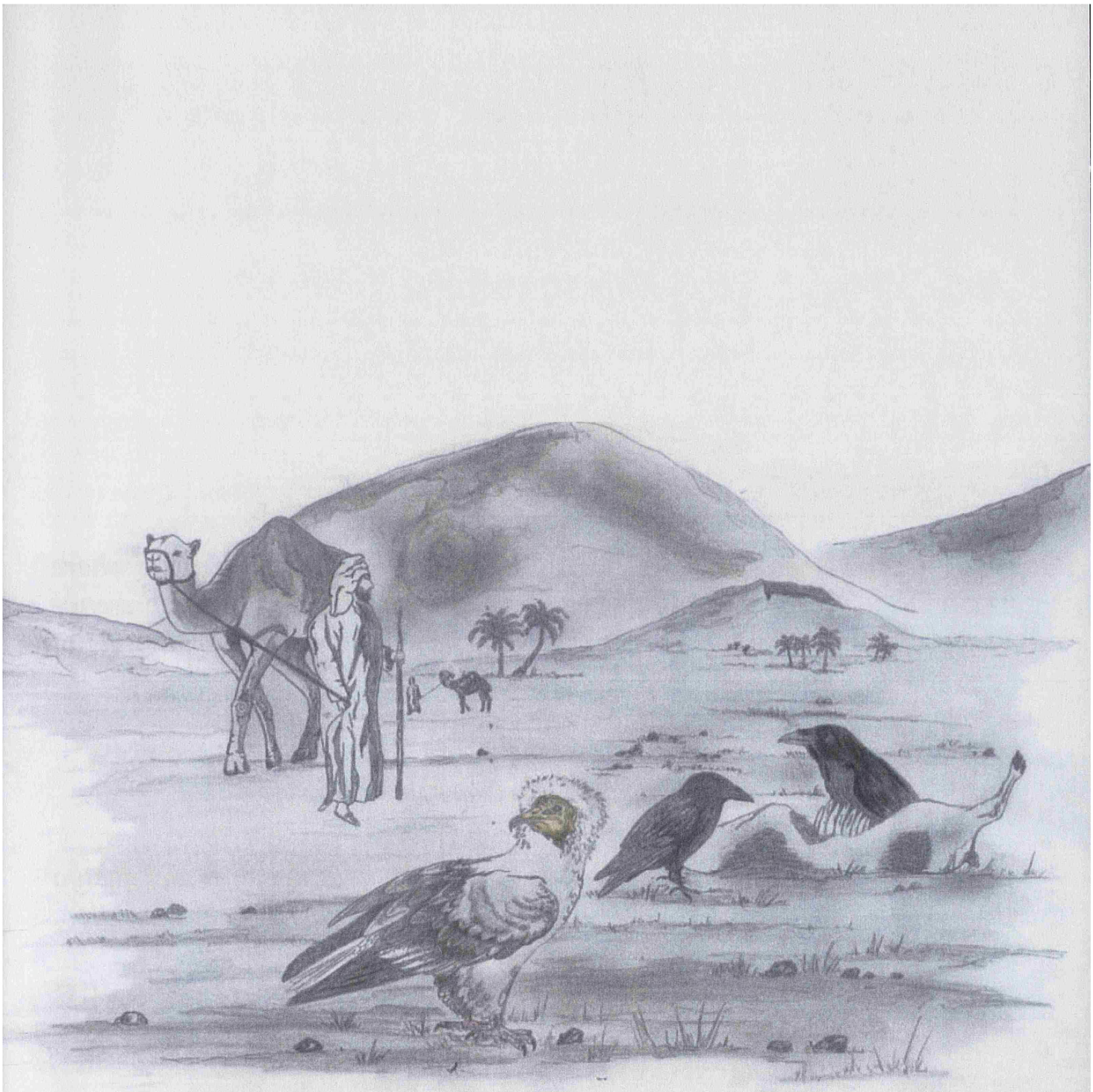


Figure S3. Neighbour-Net network of 17 MHC class II alleles (exon 2, β chain) isolated in the Egyptian vulture.



An adult Canarian Egyptian vulture stands close to a goat carcass where two crows are already feeding. He does not seem to mind of the presence of the two Berber men and their camels, inhabitants of the arid lands of Herbania.

Un Guirre adulto se acerca cauteloso al cadáver de una cabra, del que ya comen dos cuervos. La presencia de los hombres Bereberes y sus camellos, habitantes de las áridas tierras de Herbania, no parece asustarle.

CHAPTER FOUR

The Role of Humans in the Diversification of a Threatened Island Raptor

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Running title: The origin of the Canarian Egyptian vultures

El papel de los humanos en la diversificación de una rapaz insular amenazada

Resumen

Los cambios ambientales provocados por la acción humana han modificado los rangos de distribución de multitud de especies y han llevado a la extinción a otras tantas. Uno de los cuatro principales factores que han determinado la extinción de muchas especies, especialmente en el caso de las insulares, ha sido la introducción de manera directa o indirecta de formas exóticas. Sin embargo, en algunos casos, esas especies han sido beneficiosas al actuar como fuentes abundantes de alimento de las especies nativas. Por otro lado, las transformaciones que la acción humana ha llevado a cabo en el medio ambiente, han provocado de forma paralela cambios en las presiones selectivas que sufren las poblaciones y ante las que han de responder. De ese modo, determinadas adaptaciones observadas en poblaciones naturales y acontecidas en apenas siglos, han sido directamente asociadas con la actividad humana (denominada 'evolución contemporánea'). Sin embargo, pocas veces se ha cuestionado, especialmente en el caso de especies de larga vida, si esas adaptaciones surgidas en respuesta adaptativa a los cambios provocados por la acción humana, han podido promover procesos de diversificación de los taxones o incluso la posible aparición de taxones nuevos.

En este capítulo estudiamos primero la diferenciación genética y fenotípica existente entre el alimoche europeo y el canario. Después, mediante el análisis de la diversidad genética de 22 microsatélites usando dos métodos Bayesianos, datamos la colonización de la población Canaria de Alimoche y discutimos el papel del humano en dicha colonización y en su posterior diversificación. Nuestros resultados indican que es la llegada de los humanos y la consecuente llegada del ganado (cabras) a las islas, lo que permitió la colonización de los alimoches. Tras su colonización y su explosión demográfica, posible gracias a la disponibilidad de una fuente abundante y predecible de alimento (las cabras), los alimoches se diversificaron al adaptarse al nuevo ambiente insular. Esto ocurrió en apenas 200 generaciones.

Nuestros resultados sugieren que los cambios ambientales de origen antropocéntrico pueden inducir procesos de diversificación, y que esos procesos pueden tener lugar en una escala temporal ecológica, incluso en el caso de especies de larga vida.

Abstract

Background

Anthropogenic habitat modifications have led to the extinction of many species and have favoured the expansion of others. Nonetheless, the possible role of humans as a diversifying force in vertebrate evolution has rarely been considered, especially for species with long generation times. We examine the influence that humans have had on the colonization and phenotypic and genetic differentiation of an insular population of a long-lived raptor species, the Egyptian vulture (*Neophron percnopterus*).

Results

The morphological comparison between the Canarian Egyptian vultures and the main and closest population in Western Europe (Iberia) indicated that insular vultures are significantly heavier (16%) and larger (about 3%) than those from Iberia. Bayesian and standard genetic analyses also showed differentiation (F_{ST}= 0.11, p<0.01). The inference of changes in the effective size of the Canarian deme, using two likelihood-based Bayesian approaches, suggested that the establishment of this insular population took place some 2500 years ago, matching the date of human colonization. This is consistent with the lack of earlier fossils.

Conclusions

Archaeological remains show that first colonizers were Berber people from northern Africa who imported goats. This new and abundant food source could have allowed vultures to colonize, expand and adapt to the island environment. Our results suggest that anthropogenic environmental change can induce diversification and that this process may take place on an ecological time scale (less than 200 generations), even in the case of a long-lived species.

Background

The negative impact of humans on biodiversity is well known and is often referred to as 'the sixth mass extinction'. For many endangered species, humans have induced fragmentation and declines in population size that have led to strong drift in many species (Roelke *et al.* 1993; Goossens et al 1999; Snyder & Snyder 2000; Burney & Flannery 2005). Species endemic to islands have paid one of the highest tolls, as shown, for instance, by the massive extinctions that followed the human colonization of the Indo-Pacific archipelagos [5]. Human colonization of islands is typically associated with habitat destruction and fragmentation, as well as with other processes such as overexploitation or introduction of exotic species and pathogens that can seriously damage species richness (Diamond 1989; Holdaway 1999). In island ecosystems above all, invasions of exotic species have been implicated as an important factor in population loss and extinction (Wilcove *et al.* 1998; Mooney & Cleland 2001). However, alien species may also be beneficial to some native species and act, for example, as new and abundant food resources (Roemer *et al.* 2002; Gangoso *et al.* 2006).

The unprecedented rate of anthropogenic perturbation that has affected many regions during the last centuries may be directly or indirectly promoting changes in the selective forces acting on natural populations (Palumbi 2001). Consequently, human activity has become associated with evolutionary changes that occur over periods of a few hundred years, otherwise known as 'contemporary evolution' (Hendry & Kinnison 1999; Kinnison & Hendry 2001; Reznick & Ghalambor 2001). Several studies have reported adaptation occurring through contemporary evolution in species confronting anthropogenic environmental changes (see Stockwell *et al.* 2003 for a review). However, whether such anthropogenic modifications can also promote phenotypic diversification and perhaps even speciation of wild vertebrates

has rarely been considered. Nonetheless, it seems unlikely that human actions would have triggered divergent evolution in vertebrate populations, especially in those species with long generation times in which evolution is expected to proceed at a relatively slower pace than species with short generation times (Wu & Li 1985; Li *et al.* 1987).

In this study, we examine the role of humans in the origin of the phenotypic and genetic divergence of the Canarian population of Egyptian vulture (*Neophron percnopterus*). The Egyptian vulture is a long-lived trans-Saharan migratory raptor that is globally threatened (BirdLife International 2008). This vulture is one of the few raptors that has colonized islands far from continental mainlands and it has established sedentary insular populations such as the one on the Canarian archipelago. Our results demonstrate that the arrival of humans in the Canary Islands enabled the establishment of Egyptian vultures and their subsequent demographic explosion and differentiation.

Methods

Study species and populations

The Egyptian vulture (*Neophron percnopterus*) is a long-lived medium-sized scavenger bird of prey that is widely distributed throughout the circum-Mediterranean region and sub-Saharan Africa, as well as in the Middle East, Central Asia and India. Insular populations occur in the Atlantic Ocean and the Mediterranean and Arabian Seas, although many of these are now extinct (Cramp & Simmons 1980; Del Hoyo *et al.* 1994; Levy 1996). Despite its wide distribution, this vulture is globally threatened and, due to recent population declines, it is presently classified as 'Endangered' on the IUCN Red List (BirdLife International 2008). The main causes for its decline are high mortality of adult individuals caused by poisoning, collisions with wind power

turbines and electric lines, electrocution, loss of suitable habitat and food shortage due to human disturbance (BirdLife International 2008).

At present, the bulk of the European breeding population is restricted to the Iberian Peninsula (Iberia) with approximately 1500 breeding pairs (BirdLife International 2008). In the Canarian archipelago, it was very abundant in the past (Bannerman 1912), but has disappeared from five of the seven islands in recent decades (Martín 1987). Most of the Canarian population is found on Fuerteventura (the southeastern most island) where intensive monitoring over the last 12 years has revealed the presence, in average, of 30 breeding territories/year (SD=6.4). In addition, between two and four breeding pairs are usually observed every year during the breeding season on the closest island, Lanzarote, which is located less than 10 km from Fuerteventura (Donázar *et al.* 2002a; Palacios 2004, authors' unpublished data). However, these individuals are normally seen in Fuerteventura during the rest of the year, where they have been captured and banded. Other birds from Fuerteventura are occasionally observed in Lanzarote but they spend most of the time in Fuerteventura where the bulk of the population remains and more food is available.

This study is based on samples from Iberia (n=143) and from Fuerteventura (n=242) which includes approximately the 85% of the current insular population. The total population was estimated at about 200 birds in 2009 (authors' unpublished data).

Field procedures and morphological analyses

Birds were captured, ringed and sampled between 1995 and 2000 in Iberia and between 1998 and 2007 in Fuerteventura. Fledglings were captured in their nests and adult and immature birds were captured with cannon nets at supplementary feeding points in every sampled area. Birds were aged on the basis of plumage features (Cramp & Simmons 1980). All individuals were

weighed (in g) and standard body measurements were taken (in mm): length of wing chord, bill, culmen, seventh primary, tail and tarsus. To test for differences in morphological traits between the two studied populations, first we conducted a principal component analysis (PCA) of all the measured variables. Then, we performed a MANOVA test including one variable from each axis [weight (g), wing chord (mm) and bill length (mm)] and age and sex as covariates (see results for details).

Genetic analyses

Genetic diversity, population differentiation and detection of migrants

DNA was extracted from blood samples from a random subset of the individuals sampled in Iberia (n=96) and all samples available from the Canarian islands (n=242), using a standard phenol-chloroform extraction (Sambrook *et al.* 1989). Individual sex was determined in the lab by amplifying a fragment of the sex chromosomes Z and W using a polymerase chain reaction (PCR) with primers 2550F and 2718R (Fridolfsson & Ellegren 1999). The presence and size of amplification products was assessed by agarose electrophoreses. Genetic diversity was assessed using five autosomal microsatellite loci developed for the Bearded vulture (*Gypaetus barbatus*) (Gautschi *et al.* 2000) and 17 species-specific microsatellites (Agudo *et al.* 2008). We used GENALEX version 6 (Peakall & Smouse 2006) to calculate parameters of genetic variability and the differences between the two populations was tested using Wilcoxon sign-rank tests.

Population structure was measured by F_{ST} (Weir & Cockerham) that was tested for significance by performing 10,000 permutations with the programme GENETIX (Belkhir *et al.* 2004). Since this measure of differentiation/fixation is limited to some extent by the diversity of the markers (Hedrick 2005), we also calculated the standardised measure of genetic differentiation of

Hedrick (Hedrick 2005) (G'_{ST}) using SMOGD (Crawford 2010). Additionally we used the programme STRUCTURE v.2.2 (Pritchard *et al.* 2000), which employs a Bayesian clustering method to infer the most likely number of populations (K) assuming no *a priori* structure. First, we investigated the most likely K running five independent simulations of K=1-3. All simulations were run using default parameters in the admixture model and with correlated allele frequencies. Each run included 100,000 iterations of burn-in, followed by 500,000 iterations used for parameter estimation. The most likely value of K was chosen using the ΔK statistic, based on the rate of change between successive K values, as proposed by Evanno et al (Evanno *et al.* 2005). Then, non-residents or potential migrant individuals in each of the proposed clusters were identified using posterior probabilities calculated for each individual in STRUCTURE using the "usepopinfo" option.

In order to confirm the suggested migrant individuals by STRUCTURE, and detect other potential migrants from unsampled populations, we performed an assignment test implemented in GENECLASS 2.0 (Rannala & Mountain 1994; Piry et al. 2004). This program uses likelihood-based statistics in combination with resampling methods. Given that we may have not sampled all potential source populations, we used two different likelihood-based test statistics. First we estimated *Lh*, the likelihood of finding a given individual in the population in which it was sampled. This is the most appropriate statistic to use when all potential source populations have not been sampled (Rannala & Mountain 1994; Piry et al. 2004). We also used *Lh/Lmax*, the ratio of *Lh* to the greatest likelihood among all sampled populations (Piry et al. 2004), which has greater power and is most informative when all source populations have been sampled. We employed the Bayesian criterion of Rannala & Mountain (Rannala & Mountain 1994) and the resampling method of Paetkau *et al.* (Piry et al. 2004) to determine the critical value of the test statistic (*Lh* or *Lh/Lmax*) beyond which individuals were assumed to be migrants.

We selected an alpha level of 0.01 to determine critical values, as simulated data have shown this level to represent an appropriate balance between stringency and power (Piry *et al.* 2004).

Finally, to assess if the differentiation observed between the two populations could be explained without any gene flow, we used EASYPOP (Balloux 2001). This program allows simulating multilocus population datasets under a large array of conditions. We simulated two populations diverging as a result of genetic drift and without any gene flow (we performed 100 replicates). We assumed monogamy, 1000 males and 1000 females for the continental source population and 40 females and 40 males for the insular deme. Each individual in the simulation was characterized by 22 unlinked loci with a maximum of 15 alleles per locus (values as those in our dataset) with average mutation rate of $5x10^{-4}$ and 95% single step mutations (Ellegren 2004).

Demographic history

In order to estimate the date of population establishment in the Canarian archipelago, we investigated historical changes in the effective size of the Canarian population using two likelihood-based Bayesian methods. The Beaumont method (Beaumont 1999) implemented in the programme MSVAR 0.4 assumes that a stable population of size *N1* started to decrease or increase *ta* generations ago toward the current population size *N0*. The change in population size is assumed to be either linear or exponential and mutations are assumed to occur following a step-wise mutation model (SMM). Based on these assumptions and using a Bayesian coalescent-based Markov chain Monte Carlo (MCMC) approach, it is possible to estimate the posterior probability distribution of three demographic parameters scaled by the current effective population size (*N0*): $r = N0/N1$ (rate of population size change), $tf = ta/N0$ (time since the population size change started) and $\theta = 2N0\mu$, where μ is the mutation rate. Since we are testing founder

and bottleneck effects, the simulations were run under the exponential growth model. Given that this method does not allow a straight forward calculation of the time of population change (*ta*), this was calculated from *tf* after independently determining the current effective population size (*N0*) using the linkage disequilibrium method implemented in the program NEESTIMATOR (Peel *et al.* 2004). For the date calculations we estimated the species generation time (average age at which the females give birth to offspring; (Ricklefs & Miller 2000) to be around 13 years, using the data from the long-term monitoring of marked individuals (Grande 2006; Grande *et al.* 2009; authors' unpublished data).

We validated the results from the Beaumont method by obtaining another estimate of the time of population change with another method (the hierarchical model) developed by Storz and Beaumont (Storz & Beaumont 2002). This method is implemented in MSVAR 1.3 and quantifies the effective population sizes *N0* and *N1* and the time *T* (in generations) since the population size change started. It assumes an exponential change in population size and prior distributions for *N0, N1, T* and θ are assumed to be lognormal. Briefly, this method differs from the original model (Beaumont 1999) in three main aspects: 1) in the original model, multiple loci are accommodated by estimating posterior densities of the parameters for each locus separately and then taking the product of the independent densities. In the second method, posterior densities for both models are estimated using all loci in the same MCMC simulation. 2) In the original model the inferred parameters are scaled by current population size (*N0*) but in the second model the parameters are inferred separately using priors, following the approach of Tavaré *et al.* (1997) and Wilson and Balding (1998). 3) The original model is based on the assumption that all parameters other than mutation rate were identical across loci. However, in the hierarchical model parameters are free to vary from one locus to the next (for more details see (Storz & Beaumont 2002).

For both methods we used wide uninformative priors and we performed multiple runs to evaluate the stability of the estimates. The total number of iterations was larger than $2x10^8$ and thinning intervals ranged from $2x10^4$ to $5x10^4$. First 10% of the updates were discarded to avoid biases in parameter estimation due to the starting conditions as recommended by the author (Beaumont 1999). The remaining data were used to obtain the median (50%), and the lower (10%) and upper (90%) quantiles of the posterior distributions of the parameters. Consistency in the shape of the posterior distributions from the individual runs was examined to evaluate the convergence of the output values.

Results

Morphologic and genetic differentiation

The PCA extracted three main components that accounted for 77.7% of the initial variance. The first component (48.9% of the variance) included two variables with positive loadings: bill length (loading=0.88) and culmen length (loading=0.87). The second component (15.7%) clustered measurements of wing chord (loading=0.77) and primary length (loading=0.92). Finally, positive values in the third component (13.1%) were only related to weight (loading=0.90). Therefore, we performed the MANOVA test with one variable from each axis [weight (g), wing chord (mm) and bill length (mm)]. This analysis indicated an overall significant difference between populations (Wilks' Lambda= 0.52, $F_{3.265}$= 82.6, p< 0.001, Partial Eta Squared= 0.48) without effects of age and sex (p>0.05). Results showed that Canarian Egyptian vultures are significantly heavier (16%) and larger (about 3% for both wing chord and bill length) than those from Iberia (Figure 1).

Genetic analyses indicated that the Canarian population had lower genetic diversity, with an average expected heterozygosity of 0.442 and an allele richness of 2.44, than the

peninsular population, which had estimates of 0.562 and 2.98 respectively (Z=2.46, p=0.01 and Z=2.13, p=0.03). Genetic differentiation between the two populations showed that the insular and the Iberian populations were moderately genetically differentiated (F_{ST}= 0.11, p<0.01). The standardised genetic differentiation measure G'_{ST} provided a value of 0.168 indicating an important differentiation. Most microsatellite loci appeared to conform to the stepwise mutation model; four loci had at least one allele that had a length change different than the repeat unit (one base pair difference). This small portion of loci have unlikely affected our results.

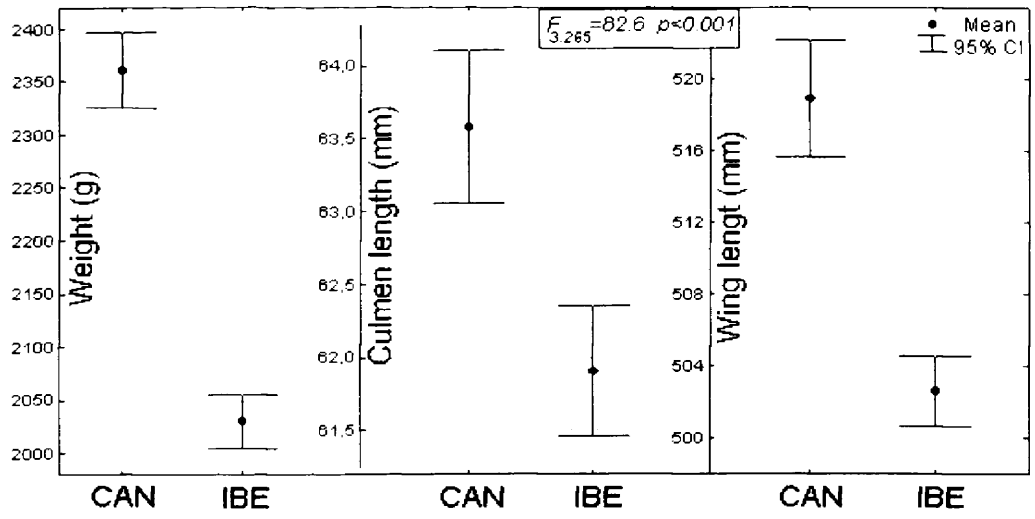

Fig 1 Mean and 95% confidence interval for three morphological traits in two vulture populations. (CA: Canary Islands, n=242; IBE: Iberian Peninsula, n=143). Results from the MANOVA test are shown.

Calculation of the statistic ΔK [37] from the STRUCTURE runs indicated that two (K=2 (ΔK=457.3; Figure 2) was the most likely number of clusters (Iberia and the Canaries, averaged of 5 runs for $Ln\ P(X \mid K)$=(-14808.16) for K=1 and (-13746.54) for K=2). All runs at K=2 produced identical clustering solutions with similar values of cluster membership q for all individuals. Almost all individuals (except for some possible migrants and their descendents, see below) from Canary Islands were assigned to their population

with q>0.85, and vultures from Iberia were assigned to a single cluster with q>0.84.

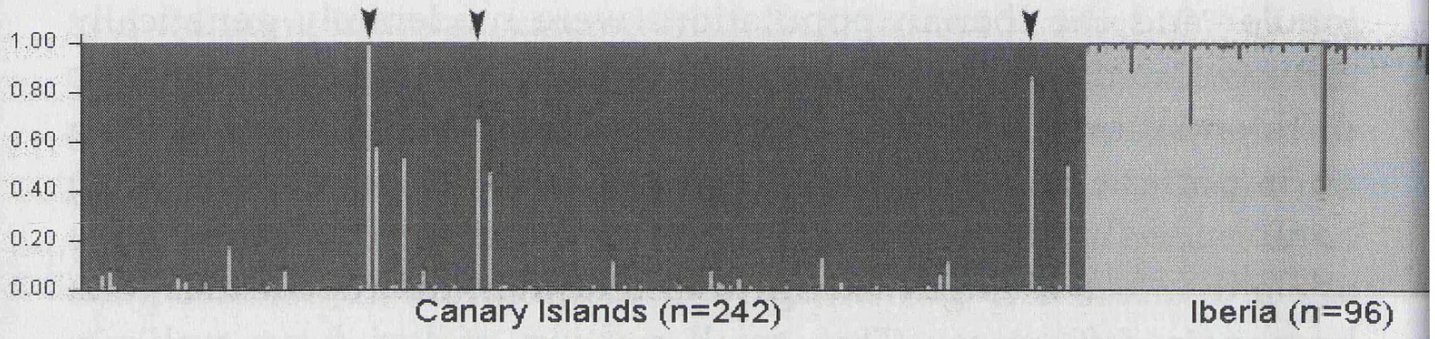

Fig 2 Clustering analysis in STRUCTURE without considering information about population of origin (k=2). Individuals are represented as vertical bars, where the amount of each colour indicates the proportion of each inferred cluster. Sampled populations are indicated (Canary Islands, N=242; Iberia, N=96). Those canarian individuals that were significantly identified as migrants (or of migrant ancestry) when using the "usepopinfo" option of STRUCTURE (see results, figure not shown), are indicated in this figure with black arrows.

Detection of migrants

Using sampling location as prior information for STRUCTURE (K= 2), we identified two individuals from the Canary Islands (06P and 035) as potential migrants (probability of membership to the Iberian population: q=0.96 and 0.823, respectively), and one individual (0R6) as potentially having migrant ancestry (q=0.48). None of the peninsular individuals seemed to have originated from the islands. Assignment tests performed with GENECLASS were concordant and also identified these individuals as migrants. We did not detect any other potential migrant individual that corresponded to an unsampled population (Table 1).

Simulation of population differentiation

In order to investigate if the differentiation observed between the two populations could be compatible with complete isolation since the colonization of the islands, we simulated how this differentiation could proceed in the absence of gene flow using EASYPOP. The simulations indicated that the two populations would need about 40 generations (over 500 years) in complete isolation to reach the F_{ST} values observed between the Canarian and the Iberian populations (0.11) (Figure 3). Given that the origin of the insular population is much older than 500 years (see below) this result indicates that occasional immigration may have contributed to limit the population differentiation.

Results from the Beaumont method suggested a strong decrease in the Canarian Egyptian vulture population size. The posterior density distribution for *log(N0/N1)* is shown in Figure 4 together with the flat prior (dotted line) for comparison, and indicates a reduction in effective population size of about three orders of magnitude (*log(N0/N1)*~-3). The posterior density of *log(ta/N0)* indicates an average value of 0.39 (10^{th}-90^{th} percentiles=0.29-0.48) (Figure 4). Time in generations (*ta*) for the population collapse was calculated by using the estimate of the current effective population size for the Canarian population calculated by the linkage disequilibrium method. This method yielded an estimation of 38.8 effective individuals (ranged from 36.1 to 41.7), which closely matched the current number of successful breeding birds (mean number of breeding pairs during the last 8 years=35, mean productivity=0.54; unpublished data from the authors). Based on this estimated effective population size, we calculated that a past population bottleneck took place around 191 generations or 2,461 years ago (median value, 10^{th}-90^{th} percentiles=2,056-2,892) and the pre-bottleneck effective population size (*N1*) was of 21,442 individuals (10^{th}-90^{th} percentiles=10,905-38,780).

Table 1 Results of the migrant detection analysis from STRUCTURE and from GENECLASS from which all individuals with probabilities of assignment to their population of origin <0.05 for either one of the two statistics (*Lh, Lh/Lmax*), are shown. Populations are: IBE: Iberia and CAN: Canary Islands.

		STRUCTURE		GENECLASS	
ID (ring)	Origin	*q* (with pop. information)	migrants F_0 [-log (*Lh*)]	Prob. pop. origin *Lh*/(*Lh*/*Lmax*)	assigned population [-log (L)]
		K=2 (IBE | CAN)			
06P		0.956 | 0.00	27.04	0.00/0.00	IBE (19.29)
035		0.823 | 0.00	19.39	0.017/0.018	IBE (19.35)
0R6		0.479 | 0.474 |	17.74	0.037/0.028	IBE (17.37)

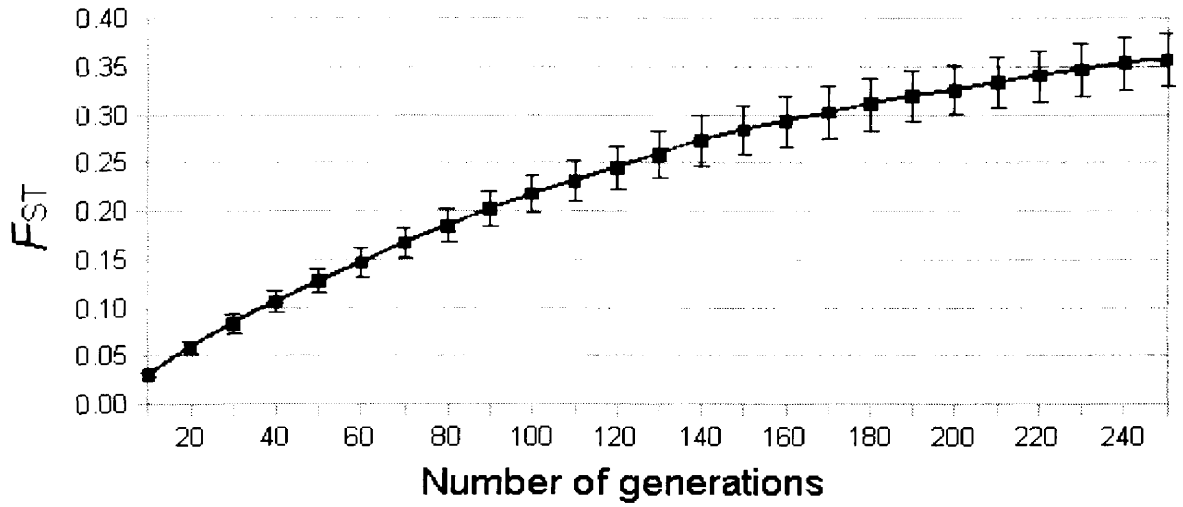

Fig 3 Increasing differentiation (F_{ST}) with time between two populations diverging by drift alone, without gene flow. Averaged F_{ST} values and standard deviation deriving from 100 replicates simulated in EASYPOP mimicking the Iberian and Canarian populations of Egyptian vultures (see text).

Estimate of size and date of population change

Results from the Storz and Beaumont method supported these findings and showed no overlap between the posterior distributions for *log(N0)* and *log(N1)*. The posterior densities were very different from the priors used (Figure 5, dashed line) and indicated a strong signature of a population bottleneck. These results suggested a past effective population size *(N1)* of 45,842 (10th-90th percentiles= 19,159-109,591), a current effective population size *(N0)* of 38 (10th-90th percentiles= 11-122) (Figure 5) and a genetic bottleneck 2,924 years ago (median value; 10th-90th percentiles= 880-9,130) (Figure 6). These results corroborate the estimates obtained with the previous approach for the current effective size and the time of the bottleneck. Although the divergence was larger between the estimates of *N1* when it was calculated with the Storz and Beaumont method, both approaches suggest a very large effective ancestral population.

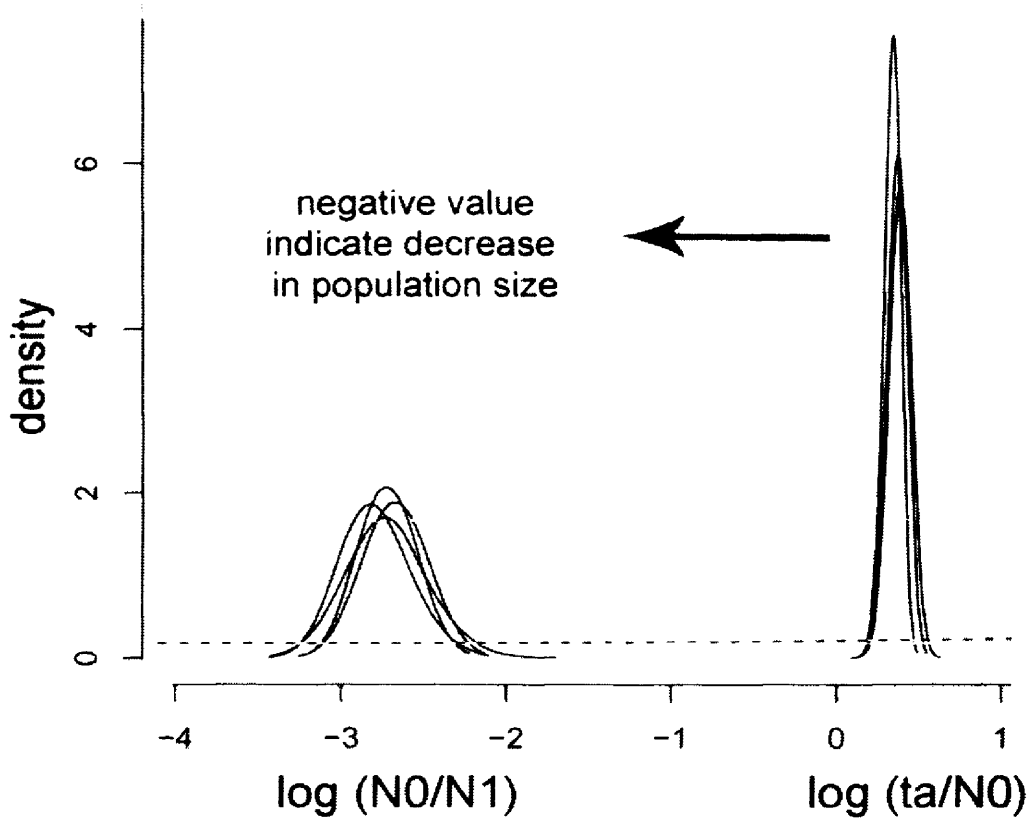

Fig 4 Population size change. Posterior distributions of the demographic parameters on a logarithmic scale obtained with the Beaumont (1999) method: (*r*) = (*N0*/*N1*) represents the ratio of present (*N0*) to past (*N1*) population size; (*tf*) = (*ta*/*N0*) represents the ratio between the time in generations (*ta*) of the population change and the present population size (*N0*). Every solid line corresponds to a different run and the prior distribution is shown for comparison (flat dashed line).

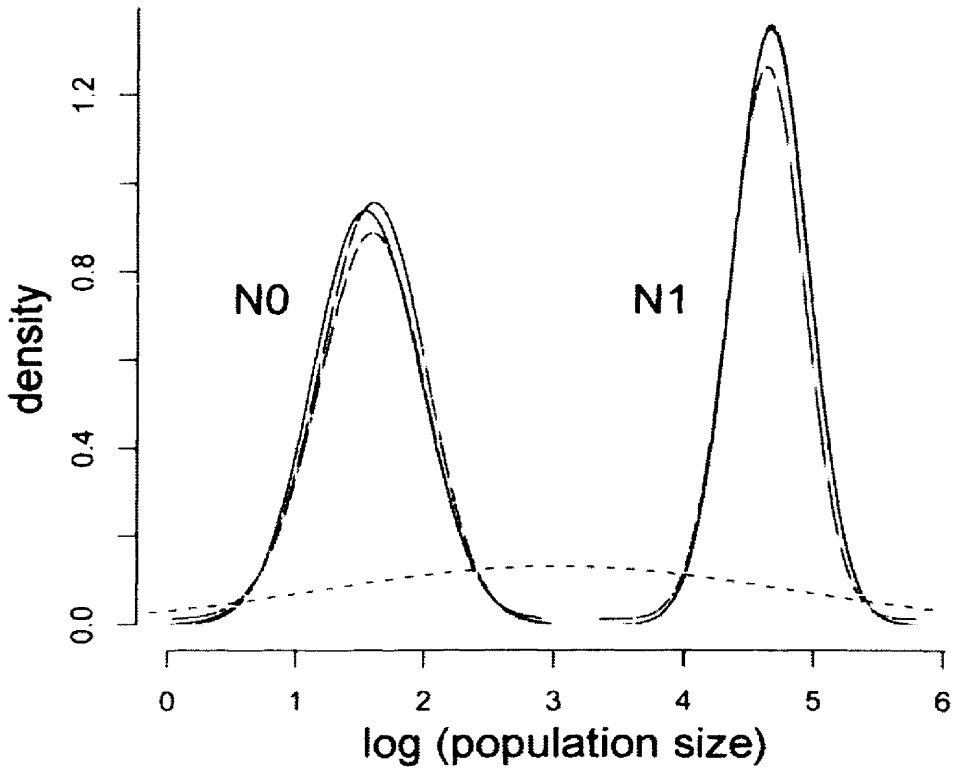

Fig 5 Ancestral and present population sizes. Posterior distributions on a logarithmic scale for past (*N1*) and current (*N0*) effective population sizes from the Storz and Beaumont (2002) method. Every solid line corresponds to a different run and prior distribution is shown (dashed line).

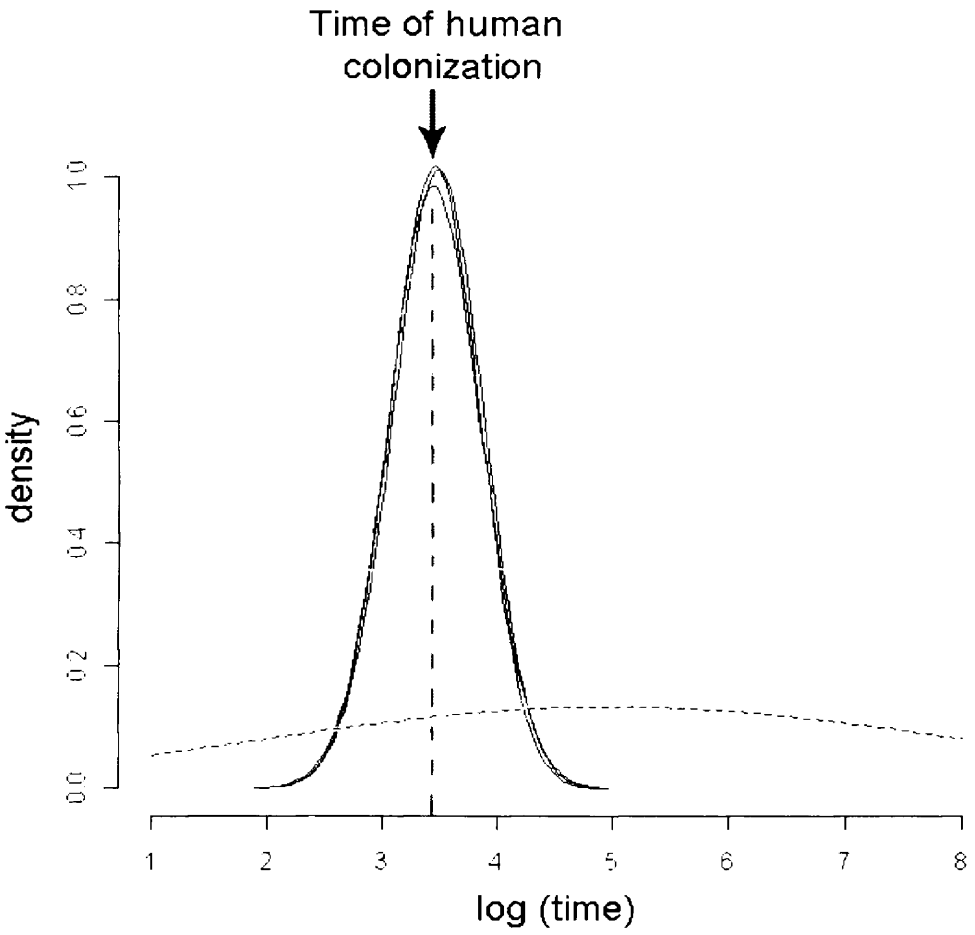

Fig 6 Time of the population bottleneck (founding event). Posterior distribution on a logarithmic scale of the date (in years) for the Canarian population founding event, obtained with the Storz and Beaumont (2002) method. Every solid line corresponds to a different run. The prior is shown as a dashed line with median 100,000 years ago and the arrow corresponds to the date of human colonization as indicated by the archaeological record (about 2,500 years ago).

Discussion

The Bayesian analysis of the historical demography of the Canarian Egyptian vulture population revealed the existence of a bottleneck approximately 2500 years ago and an ancestral effective population of tens of thousands of individuals. It is unrealistic to assume that this estimate represents the former Canarian population for which, moreover, no fossil evidence exists. Even though the fossil chronology of the Quaternary Period is well preserved and birds are one of the best represented groups, especially in Fuerteventura (Cabrera 1996), the only remains of Egyptian vultures appear to be modern (Jaume *et al.* 1993). This would suggest that the species was rare or absent from the islands until recently. The absence of large terrestrial mammals could well have precluded the successful colonization of the islands by large scavengers. Food resources available to vultures in the Canary Islands before the arrival of domestic animals were scarce and variable since they would have consisted only of the remains of seabirds and sea mammals, or of rodents (Cabrera 1996; Bocherens *et al.* 2006). Even though shoreline carrion is a valuable resource for some vulture populations (Chamberlain *et al.* 2005), it is probably not sufficient for maintaining a stable reproductive population in islands as small as those of the Canarian archipelago.

It is thus more likely that the estimated effective population size of tens of thousands of birds corresponds to the ancestral source population from which the founders of the Canarian population originated. Consequently, the date of the bottleneck suggested by the genetic data would correspond to the date the insular population was established, which closely matches the arrival of the human colonizers of these islands about 2500 years ago (Cabrera 1996). Archaeological remains show that these first inhabitants were Berber people from northern Africa, who imported and maintained herds of goats (*Capra hircus*).

Subsequent chronicles dating from the European conquest in the fifteenth century describe large numbers of goats, with more than 60,000 on Fuerteventura (1659 km^2) (Cabrera 1996). Hence, the arrival of humans and subsequent livestock could have provided sufficient food resources to enable colonization by Egyptian vultures. In historical accounts from the sixteenth to twentieth centuries, these birds are described as very abundant and dependent on domestic animals (Bannerman 1912, 1963; Cabrera 1996).

The introduction of this new and abundant food source by humans could have allowed not only the colonization by these vultures, but also their demographic expansion and their putative adaptation to the new island environment. The phenotypic differences observed between the Canarian Egyptian vultures and their potential source population (Iberia) may be due to drift, resulting from the isolation and small effective population size in the islands. However, some morphological and ecological changes observed in the insular vultures are compatible with various characteristic features associated with insularity: Canarian birds are sedentary (Carlquist 1974), exhibit tendency to gigantism (Sondaar 1977) and are tamer (Gangoso & Palacios 2005).

Our genetic analyses reveal clear divergence and support the current classification of the insular deme as a separate subspecies (*N. p. majorensis*) (Donázar *et al.* 2002b). This differentiation indicates that admixture between the Iberian and Canarian populations may be rare. However, the finding of two immigrant Iberian birds and one individual of admixed ancestry on the islands substantiates the fact that, like many other trans-Saharan European species (Martín & Lorenzo 2001), Egyptian vultures occasionally reach the archipelago. The migratory route of these vultures crosses the Western Sahara desert and can, on occasions, pass along the West African coast, only 95 kilometres away from the Canary Islands and a crossable distance for this species (Cramp & Simmons 1980; authors' unpublished data).

These observations suggest that, although Iberian Egyptian vultures could have been able to regularly reach this archipelago, they were unable to establish a stable population until the arrival of humans and goats.

This unique Canarian Egyptian vulture population has suffered a precipitous decline during the second half of the twentieth century caused by mortality due to human persecution (Donázar *et al.* 2002a). However, food availability has never been a concern for the conservation of the species in the islands (Donázar *et al.* 2002a; Gangoso *et al.* 2009). Goat-raising is still the most important economic activity in Fuerteventura and goat carcasses are still this species' primary source of food (Gangoso *et al.* 2006). It is paradoxical that while human activities are behind the origin of this divergent lineage, other human activities are contributing to its demise.

Conclusions

The bottleneck associated with the colonization of the Canarian archipelago (followed by demographic expansion), together with the presumably different selective pressures of a new environment, may have promoted diversification in this species, which has occurred over less than 200 generations. Therefore, our results show that anthropogenic environmental changes can induce vertebrate diversification and that this process can take place on an ecological time scale, even in the case of long-lived species.

An adult Egyptian vulture takes care of its fledgling lying on the nest that was built inside a small cave on a cliff, with sticks, wool and pieces of clothing found somewhere

Un Alimoche adulto vigila a su volantón que descansa en el nido, construido en el interior de una cueva en la pared de un acantilado, con palos, lana y trozos de tela encontrados en cualquier lugar.

CHAPTER FIVE

Genetic Diversity at Neutral and Adaptive Loci determines Individual Fitness in a Long-lived Territorial Bird

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Running title: Heterozygosity Fitness Correlations in Canarian Egyptian vultures

La diversidad genética en genes neutrales y adaptativos determina la eficacia individual en un ave territorial de larga vida

Resumen

La depresión por endogamia es la pérdida de 'salud' debido al incremento en la expresión de alelos deletéreos o perjudiciales (generalmente recesivos) y la pérdida de heterozigosidad en genes funcionalmente importantes. La endogamia afecta a la supervivencia de las poblaciones a medio y largo plazo. Por ese motivo uno de los principales objetivos de la biología de la conservación es entender los mecanismos que promueven la pérdida de diversidad genética y el efecto de dicha pérdida en las poblaciones. La mayoría de los estudios que abordan este problema en poblaciones naturales, lo hacen empleando medidas indirectas, usando generalmente marcadores neutrales (como por ejemplo la heterozigosidad individual). Esto es debido a que obtener valores precisos de endogamia individual supone disponer de pedigríes completos de varias generaciones, lo que suele ser en la mayoría de los casos, imposible. Sin embargo, se ha criticado y aun hoy está en debate, la adecuación del empleo de estas medidas indirectas debido a que no se sabe hasta que punto la heterozigosidad medida en varios loci neutrales representa la heterozigosidad genómica de un individuo. Por este motivo, es muy recomendable analizar de forma paralela, genes con significado evolutivo. Desgraciadamente pocos genes funcionales han sido caracterizados en las especies salvajes y están disponibles para su uso en las poblaciones naturales. En ese sentido y como ya se ha explicado en el capítulo 3, los genes del MHC son una excepción. El empleo de estos genes en la investigación de la depresión por endogamia tiene además mucho sentido ya que están directamente relacionados con la capacidad inmune de los individuos, y por lo tanto con su salud y su supervivencia.

El objetivo principal de este capítulo es desentrañar el papel que la diversidad neutral y funcional juega sobre la salud individual en una población aislada de un vertebrado de larga vida; el alimoche canario o guirre. Para abordar este objetivo comparamos los valores de diversidad genética obtenidos tras el análisis de los 22 microsatélites y dos loci del MHC clase II β, con dos parámetros de salud individual: éxito reproductor y edad de reclutamiento (edad de la primera reproducción). Nuestros resultados indican que existe una relación negativa entre la heterozigosidad individual (como una medida indirecta de la endogamia) y la edad de reclutamiento. De este modo los individuos con menor 'endogamia' comienzan antes la reproducción. Por otro lado observamos que una vez que los individuos comienzan a reproducirse, es la variabilidad en los genes del MHC lo que determina un mayor éxito. Éste viene determinado por un lado por la diversidad en el MHC (número de alelos) y por otro lado, por la posesión de determinadas combinaciones de alelos. Nuestros resultados demuestran que los guirres presentan depresión por endogamia, es decir que la pérdida de diversidad genética provocada por el declive poblacional está teniendo un efecto significativo en la salud individual y en la dinámica poblacional. Por un lado, está afectando a la edad de reclutamiento, lo que puede tener consecuencias graves en la demografía poblacional, dada la elevada mortalidad no natural a la que están sujetos los adultos de esta población. Por otro lado, la diversidad del MHC está afectando a la productividad de los adultos reproductores. Como hemos visto en el capítulo 3, el incremento exponencial en la riqueza y la abundancia de patógenos observado en Canarias en la última década se ha visto acentuado por la menguada capacidad de respuesta inmunitaria de los guirres. De este modo, la concatenación de los factores promotores del declive de esta población y de los factores asociados a la insularidad y a un reducido tamaño poblacional, podrían tener consecuencias catastróficas en la supervivencia de esta subespecie única a corto-medio plazo.

Abstract

There is compelling evidence about the manifest effects of inbreeding depression on the individual fitness and the populations' risk of extinction. The majority of studies addressing inbreeding depression on wild populations are generally based on indirect measures of inbreeding using neutral markers. However, the study of functional loci, as genes of the Major Histocompatibility Complex (MHC) is highly recommended. MHC genes genes constitute an essential component of the immune system of individuals, which is directly related to individual fitness and survival. In this study, we analyze heterozygosis fitness correlations of neutral and adaptive genetic variation (22 microsatellite and two loci of MHC Class II, respectively) with the age of recruitment and breeding success of a decimated and geographically isolated population of a long-lived territorial vulture. Our main results indicate a negative correlation between neutral genetic diversity and age of recruitment. This finding suggests that inbreeding may be delaying reproduction which in turn, may reduce the number of breeding opportunities and the probability of an individual to acquire experience and hence to succeed. Secondly, we found a correlation between genetic diversity at MHC genes and breeding success. Globally, our findings demonstrate that the genetic depauperation in small populations has a negative impact on the individual fitness, thus increasing the populations' extinction risk.

Introduction

Inbreeding depression is the decline in mean fitness due to the increase expression of recessive deleterious alleles and the loss of heterozygous advantage at functionally important genes (Charlesworth & Charlesworth 1987). There is now compelling evidence about the effects of inbreeding on the performance of individuals by reducing their survival, breeding success and resistance to environmental stress (Crnokrak & Roff 1999; Keller and Waller 2002; Armbruster & Reed 2005), therefore contributing to enhance the risk of extinction of natural populations (e.g. Madsen *et al.* 1996, Lacy & Horner 1997, Acevedo-Whitehouse *et al.* 2003, Liberg *et al.* 2005; O`Grady 2006).

Effects of inbreeding in the wild should be ideally assessed by estimating individual inbreeding coefficients from detailed pedigrees. However, this information is inherently difficult to obtain specially in long-lived species. Consequently, a large number of studies have used molecular markers to get indirect estimates of inbreeding and many have found significant heterozygosity-fitness correlations (HFCs) (e.g. Coltman *et al.* 1998; Thelen & Allendorf 2001; Foerster *et al.* 2003; Hoffman *et al.* 2004; Markert *et al.* 2004; Charpentier *et al.* 2005; Da Silva *et al.* 2006; Hanski & Saccheri 2006; Brouwer *et al.* 2007; Jamieson *et al.* 2007; Ortego *et al.* 2007; Grueber *et al.* 2008; Luikart *et al.* 2008). Two main hypothesis are proposed to explain HFCs: i) the *local effect hypothesis* suggest that neutral loci can be linked to loci influencing fitness and hence heterozygotes would exhibit heterozygote advantage by two main processes: dominance, i.e. heterozygotes experience lower expression of recessive deleterious mutations, and overdominance, i.e. heterozygotes are superior *per se* (Hill & Robertson 1968; Otah 1971; David 1998; Tregenza & Wedell 2000; Hansson & Westerberg 2002), ii) the *general effect hypothesis* suggest that multilocus heterozygosity is reflecting genomic-wide variation or inbreeding (Weir and

Cockerham 1973; David 1998; Acevedo-Whitehouse *et al.* 2005; Luikart *et al.* 2008; Da Silva *et al.* 2009). However, to what extent heterozygosity at a few neutral loci reflects genome-wide diversity remains, nonetheless, controversial (Balloux *et al.* 2004; Slate *et al.* 2004; Hansson & Westerberg 2008; Chapman *et al.* 2009). A recent review supports the use of HFCs but recommends the use of a comprehensive panel of markers and large sample sizes (Szulkin *et al.* 2010). This and other studies also suggest that heterozygosity correlation among markers (identity disequilibrium or ID) may indicate that their averaged heterozygosity is likely to be informative about inbreeding (Pemberton 2004; Aparicio *et al.* 2007; Hansson & Westerberg 2008; Szulkin *et al.* 2010). Nevertheless, correlations between neutral genetic diversity and fitness still remain weak and inconsistent and are expected to depend on population demography and life history (Coltman & Slate 2003; Hansson & Westerberg 2002; Chapman 2009). Therefore, genetic studies on population viability should also ideally analyze adaptive loci (Frankham 2010).

Genes of the Major Histocompatibility Complex (MHC) are good candidates for these kinds of studies. These genes are known to play a crucial role during pathogen confrontation and clearance and their variation is thought to determine the capability of individuals to respond to pathogens and parasites, and consequently, MHC diversity has been traditionally associated with individual fitness and outcome of infection (reviewed by Oliver *et al.* 2009; Radwan *et al.* 2009; Spurgin & Richardson 2010). Vertebrates have at least two classes of MHC loci, all of which code for membrane-bound proteins central to the immune response. Class I loci recognize intracellular pathogens such as viruses and Class II loci are involved in responses to extracellular pathogens such as bacteria and fungi. MHC loci are clearly under selection, and provide a useful tool with which to investigate adaptive variation in vertebrates and its relationship with fitness (Sommer 2005, Piertney & Oliver 2006).

Many long-lived vertebrate species are currently highly threatened due to their conservative life-history strategies which determine their vulnerability to human-related pressures (Groombridge & Jenkins 2002). The maintenance of their populations is important from an ecological point of view, since they used to be key species for ecosystem's functioning (e.g. top predators) and because they can act as umbrella species (i.e. their preservation enables the parallel conservation of other threatened species with lower social charisma) (Sergio *et al.* 2006). The conservation of insular populations of long lived organisms is especially relevant given the habitual phenotypic, genotypic and ecological differences of these entities. Furthermore, these populations are especially vulnerable to inbreeding and inbreeding depression because they are naturally reduced and genetically depauperate (Pimm *et al.*, 1988, Frankham, 1995), and accordingly, they are usually highly threatened and present higher extinction rates (Frankham, 1998). Obtaining individualized information necessary for the proper management of these populations is though difficult and involves long-term monitoring. For this reason there are few studies addressing inbreeding depression in threatened populations of long-lived species (e.g. Charpentier *et al.* 2005, 2008; Da Silva *et al.* 2006; Kretzmann *et al.* 2006; Luikart *et al.* 2008; Blomqvist *et al.* 2010), much less including the analysis of functional genes (e.g. Da Silva *et al.* 2009; Banks *et al.* 2010; Worley *et al.* 2010).

In this study we investigate the occurrence of inbreeding depression in a highly reduced and potentially inbred insular population of a globally endangered bird of prey, the Egyptian vulture (*Neophron percnopterus).* For this purpose we explore HFCs by relating neutral (using 22 microsatellites) and functional (genes of the second exon of MHC class II β) genetic diversity with two demographic parameters known to greatly affect the individual fitness: age of recruitment into the breeding population and breeding success (Cole, 1954; Lewontin, 1965; Caswell & Hastings,

1980; Oli & Dobson, 1999; Weimerskirch 1992; Congdon *et al.* 1993; Saether & Bakke 2000; Eberhardt 2002; Becker *et al.* 2007; Grande *et al.* 2009; Sergio *et al.* 2009; Blas *et al.* 2009). Inbreeding depression is most prominent for characters associated with reproductive fitness (Falconer & Mackay 1996; Lynch & Walsh 1998), as age of recruitment. With respect to this parameter, divergent individual reproductive strategies implying dissimilar trade-offs, may be observed within a population: i) breeding earlier may increase fitness by raising the probability of realizing reproduction and reproductive experience (Cam & Monnat 2000; Barbraud & Weimerskirch 2005; Sergio *et al.* 2009; Blas *et al.* 2009; Aubry *et al.* 2009) but accelerate, conversely, the effects of aging. ii) delaying maturity permits better survival and additional growth or experience thus increasing future reproductive output (Oli *et al.* 2002; Charmantier *et al.* 2006; Aubry *et al.* 2009), but reduces the number of breeding opportunities, the probability to acquire experience before dying (Blas *et al.* 2009), and the fitness due to longer generation distance (Wooller *et al.* 1990, 1992; Oli et al. 2002). For long-lived species, previous studies have suggested strong directional selection for early maturity (e.g. Oli *et al.* 2002; Blums *et al.* 2002; Charmantier *et al.* 2006) and lower lifetime productivity in animals recruiting at older ages (see Becker *et al.* 2007 for a review).

We therefore specifically test the following predictions: i) younger recruits correspond to the higher quality individuals and hence they are more heterozygous and less inbred ii) there is a positive correlation between breeding success and heterozygosis and iii) regarding to MHC variability and given that these genes are under selection, we expect that higher frequent genotypes will be advantageous for individuals compared to rare genotypes.

Methods

Studied species and population

The Egyptian vulture is a long-lived and medium-sized (2kg) scavenger that presents a broad distribution range within the dry areas of Europe, Asia and Africa and holds insular populations in the Atlantic Ocean and the Mediterranean and Arabic Seas. It is a trans-Saharan migratory raptor but insular populations are sedentary. Reproductive individuals maintain exclusive breeding territories during the breeding season when they nest on cliffs and usually lay two eggs (Cramp & Simmons 1980, Donázar 1993). The species is declining and threatened in all of its distribution range as a consequence of non-natural mortality (Carrete *et al.* 2007; BirdLife International 2008). At present, only 30,000 to 40,000 mature individuals survive in the whole world and this vulture is considered "Endangered" (BirdLife International 2008). Insular populations have also suffered drastic declines or even becoming extinct in some cases (Levy 1996; Sarà *et al.* 2009; Gangoso *et al.* 2006). The present study is based on one of the main insular populations that still remain in Western Europe; the Canarian population. There the species is described as a differentiated subspecies (*N. p. majorensis*) (Donázar *et al.* 2002a) and whereas it was very abundant in the past (Martín, 1987, Donázar *et al.* 2002b, Palacios 2004, Agudo *et al.* 2010), has also suffered a precipitous decline during the second half of the XXth century (Martín 1987). Therefore, at present most of the Canarian population is relicted to Fuerteventura (the south easternmost island) where intensive monitoring over the last twelve years has revealed the presence, in average, of 30 breeding territories/year (SD=6.4) with a mean productivity (number of fledglings/ number of breeding pairs) of 0.53 (SD=0.05). In addition, between 2 and 4 breeding pairs are usually observed every year during the breeding season on the closest island, Lanzarote, which is located

less than 10 km from Fuerteventura (Donázar *et al.* 2002b; Palacios 2004; authors' unpublished data). The total population was estimated at about 200 birds in 2009 (authors' unpublished data).

Monitoring of individual fitness components

From 1998 to 2009, 175 fledglings were captured at nests and 82 immature and adult birds were trapped by cannon netting. All birds were marked with both metal and plastic rings with an individual alphanumeric code. Adult and immature birds were aged on the basis of plumage features (Cramp & Simmons 1980). We estimated that in 2009 85% of the population was individually marked. During the same period, we monitored the breeding population by visiting occupied territories (n=38 territories in 2009) during the breeding season (from late February to late June) to determine the presence and identity of breeders, as well as the breeding success (for details, see Grande *et al.* 2009).

Genetic amplification and genetic tagging of not banded individuals

DNA extractions were performed from blood samples using a standard phenol-chloroform extraction (Sambrook *et al.* 1989). Individual sex was determined in the lab by performing the polymerase chain reaction (PCR) with primers 2550F and 2718R (Fridolfsson & Ellegren 1999) on DNA extracts obtained from blood samples. Individual genetic diversity at neutral loci and kin relationships were calculated using the genotypes previously obtained by the authors using 22 microsatellite loci (Agudo *et al.* 2008; Agudo *et al.* 2011). Naturally moulted feathers from non-banded adult birds were also collected at breeding territories. Genomic DNA from the blood clot contained within the feather shaft was extracted according to Horváth *et al.* (2005). DNA extracted from feathers has been successfully used in previous

studies of population genetics and parentage analysis (Martinez-Cruz *et al.* 2007; Rudnick *et al.* 2005, Alcaide *et al.* 2010). DNA samples from feathers were also genetically sexed (Fridolfsson & Ellegren 1999) and genotyped for the 22 microsatellite loci. We used the program GENALEX 6.2 (Peakall & Smouse 2006) for detecting repeated genotypes and assigning genetically profiled feathers to individual adults. We successfully amplified and sexed 135 feathers (<10% of feathers did not amplified) that yield 26 new (not banded) breeding individuals. Feathers from territories occupied by banded birds matched in 99% with already resolved genotypes. This finding suggests that the collection of moulting feathers from other adult birds rather than the territorial ones is unlikely.

Paternity assessment

Parentage was evaluated through both field observations and the genotypes of the 22 microsatellite loci (Agudo *et al.* 2011) using the likelihood-based approach implemented in CERVUS version 3.0 (Kalinowski *et al.* 2007). This method calculates statistical confidence based on the difference in LOD (i.e., the logarithm of the likelihood ratio) scores of candidate parents. This confidence is determined using criteria that are generated through simulation where the proportion of candidate parents sampled, the percentage of missing genotype data and sampling errors are taken into consideration (values were set by default at 0.75, 0.01 and 0.01, respectively). This molecular method allowed us to corroborate field information, resolve the kinship of individuals with unknown parents, i.e. those that were captured as immature of adults, identify cases of extra pair paternity and resolve the identity of the parents in the case of breeding trios (n=4).

Neutral genetic diversity and genealogical relationships

Individual genetic diversity at neutral loci was estimated in for 242 individuals by estimating two different measures homozygosity by loci (*HL*) and internal relatedness (*IR*) using CERNICALIN (Aparicio *et al.* 2006; Ortego *et al.* 2007) . *HL* is though to improve heterozygosity estimates by weighting the contribution of each locus to the homozygosity value depending on its allelic variability. *IR* is based on allele sharing and the frequency of every allele counts towards the final inbreeding value, being shared rare alleles more heavily weighted than shared common alleles (Amos *et al.* 2001). Both metrics were calculated. We selected these two measures as indirect indices of inbreeding because they have been suggested to be better predictive of pedigree inbreeding coefficient than d^2 (i.e. multilocus estimator of the squared difference in microsatellite allele sizes within and individual (Goldstein *et al.* 1995)). Furthermore, theoretical and practical studies have shown that heterozygosity outperforms d^2 in estimating relationships between genetic variability and fitness in all but a few circumstances, and therefore d^2 must be of limited use (Keller & Waller 2002). Finally, we calculated maximum-likelihood estimates of pairwise relatedness coefficients and genealogical relationships from multilocus genotypes with the method implemented for microsatellite data in the software ML-RELATE (Kalinowski *et al.* 2006).

Test of Identity Disequilibrium

To test whether heterozygosity at our panel of neutral markers actually reflects genome-wide heterozygosity, we measured the identity disequilibrium (ID), i.e. the correlation in heterozygosity across loci, by calculating the parameter g_2. This parameter is a measure of the excess of double heterozygotes at

two loci relative to the expectation under a random association (i.e., covariance in heterozygosity), standardized by average heterozygosity. Under any form of inbreeding, this measure should be constant whatever the pair of loci considered (Szulkin *et al.* 2010). We calculated g_2 using the software RMES (David *et al.* 2007) which also tests whether g_2 differs significantly from zero based on 1000 iterations.

Functional genetic diversity

Functional genetic diversity was assessed by amplifying the second exon of MHC class II β genes using capillary Single Strand Conformational Polymorphism (SSCP) analyses (Agudo *et al.* in press). For every individual, we obtained the allele composition and the number of alleles (from two to four alleles).

Heterozygosity Fitness Correlations

We used Generalized Mixed Models (GLMMs) to explore the relationships between neutral genetic diversity estimates (*IR* and *HL*), between neutral and functional genetic diversity (both GLMMs with normal error distribution and identity link function) and finally between genetic diversity and the fitness components. Age of recruitment into the breeding population (log-transformed, normal error distribution and identity link function) was modelled by including as explanatory variables individual's sex, homozygosity (*HL*), internal relatedness (*IR*), MHC genotype, and number of different MHC alleles. As reproductive parameters can show inter-annual variability, we included "year" as a random term in models. The relationship between the breeding success (number of successful reproductions / number of reproductive attempts; binomial error distribution and logit link function) and genetic diversity was assessed at the individual level and then by considering the breeding pair as sampled units. The first models

included individual's age and sex and variables describing neutral (*HL, IR*) and functional (MHC genotype and number of different MHC alleles) genetic diversity. The second models considered individual's age, *HL, IR,* and MHC genotype of each mate, total number of different alleles in the breeding pair, and the parentage coefficient between the mates Full models were simplified by removing terms with $p > 0.1$(backward procedure, Zuur *et al.* 2009) and significance level was established at $p<0.05$.

Results

Neutral and functional genetic diversity

The average value for individual microsatellite homozygosity (*HL*) and internal relatedness (*IR*) were 0.40 (SD=0.12) and 0.01 (SD=0.18) respectively. Measures of *HL* and *IR* were highly correlated ($P < 0.0001$; $r = 0.89$). Results of the analyses of identity disequilibrium also indicated a significant correlation in heterozygosity across loci (g_2 =0.007, SD=0.005, p=0.03). Pairwise relatedness coefficients between mates ranged from 0 to 0.63 with an average value of 0.07 (SD=0.11). This value was similar to the average value within the whole population (0.08, SD=0.13).

Out of 236 birds, 103 individuals presented two MHC class II alleles, 19 birds presented three different alleles and 114 had four alleles. Overall, ten different alleles that comprised a total of 9 different genotypes were distinguished, with three genotypes (G1-G3, Table 1) accounting for 89% of the genotypic variation in this population (Table 1).

Table 1 Allele composition and frequencies of the 9 different genotypes of the MHC class II β, found in the Canarian Egyptian vultures (from Agudo *et al.* in press; Chapter 3)

Alelle 1	Alelle 2	Allele 3	Allele 4	Geno -type	Allele Diver- gence	Frequen cies
Nepe18	Nepe19			G1	36	0.259
Nepe1	Nepe5			G2	51	0.174
Nepe18	Nepe19	Nepe1	Nepe5	G3	79	0.436
Nepe18	Nepe19	Nepe1	Nepe6	G4	81	0.017
Nepe1	Nepe5	Nepe6		G5	80	0.009
Nepe1	Nepe7	Nepe5		G6	75	0.076
Nepe1	Nepe7			G7	48	0.004
Nepe1	Nepe20	Nepe7		G8	68	0.004
Nepe1	Nepe5	Nepe8	Nepe9	G9	79	0.017

Neutral and functional genetics were negatively linked, as shown by the significant relationship between both measures of homozygosity and the number of MHC alleles (Fig. 1).

Heterozygosity Fitness Correlations

Age of recruitment into the breeding population ranged from 4 to 10 years old (average = 5.8, SD=1.45, Fig. 2). Earlier recruits (individuals starting breeding at four to five years old) presented significantly lower levels of internal relatedness than those recruiting later (from six to ten years old). Although marginally significant, a similar relationship was also found between recruitment and *HL* (p=0.05). However, when simultaneously included in models, just *IR* remain significant (p=0.04), *HL* loosing even more its significance (p=0.10). We did

not find effects of sex (p=0.38) and functional genetic diversity (all p>0.5) (Table 2).

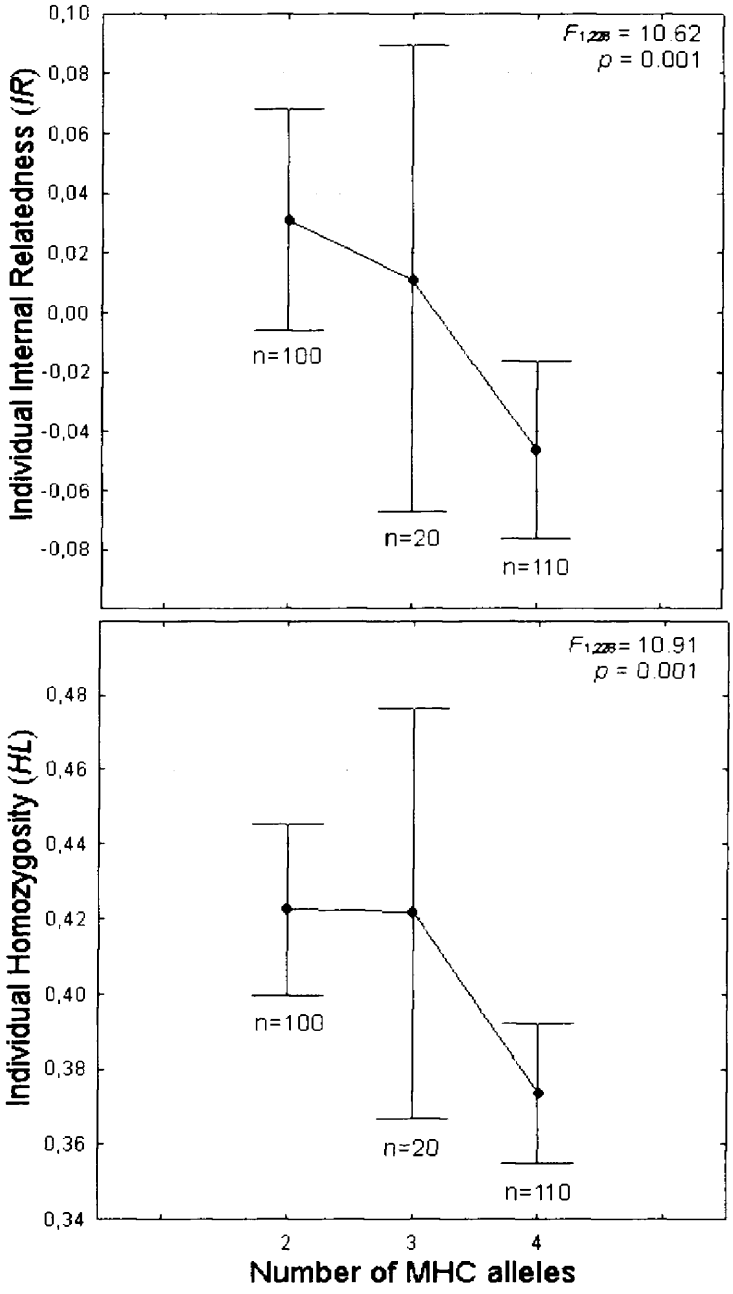

Fig 1 Average values and 95% confidence intervals of individual internal relatedness (above) and homozygosity (below) in Canarian Egyptian vultures with two, three and four different MHC alleles.

Table 2 GLMs for age of recruitment (log-transformed; link function: identity, error distribution: normal), individual breeding success (number of successful reproductions/number of reproduction attempts; link function: logit, error distribution: binomial) and breeding success of pairs (link function: logit, and error distribution: binomial) in Canarian Egyptian vultures. Only variables retained in the models are indicated.

Model	Variable	Num. DF	Den. DF	*F*	*p*
Age of recruitment	*IR*	1	37	5.16	0.0290
	HL	1	37	3.96	0.0541
Individual breeding success	MHC	7	91	3.55	0.002
	No. Diff. Alleles	1	97	5.55	0.020
Breeding success of pairs	MHC*f*	5	33	4.77	0.0022
	No. Diff. Alleles	1	77	15.93	0.0001

During the study period, we controlled 393 reproduction attempts. Only 37.7% of the observed breeding attempts were successful, most of them resulting in a single fledgling (94.6%). The breeding success of the individuals was significantly linked to functional genetic diversity but not to neutral one (all $p>0.3$) (Table 2). The Tukey-Kramer analyses for multiple comparisons indicated that individuals with genotype G3 presented higher success than individuals with genotypes G2 and G4, while birds with genotype G6 had significant higher success than birds with genotype G4 (Fig. 3). Moreover, individuals with higher numbers of different alleles in their MHC genes have higher breeding success than those with lower number of alleles ($F_{1,97}=5.55$, $p=0.02$).

Fig 2 Distribution of age of recruitment in the Canarian population of Egyptian vultures showing the neutral genetic variability (internal relatedness, *IR*, black dots, and homozygosity by loci, *HL*, grey dots) of individuals within each class.

When focusing on the breeding success of breeding pairs, we found that those composed by individuals holding a higher number of different alleles were more successful at breeding than pairs formed by genetically similar birds ($F_{1,77}$=15.93, p=0.0001). We also detected a significant effect of the female MHC genotype on the breeding success ($F_{5,33}$=4.47, p=0.002), while that of males was not significant. As in the previous analysis, neutral genetic diversity was not linked to the breeding success of breeding pairs.

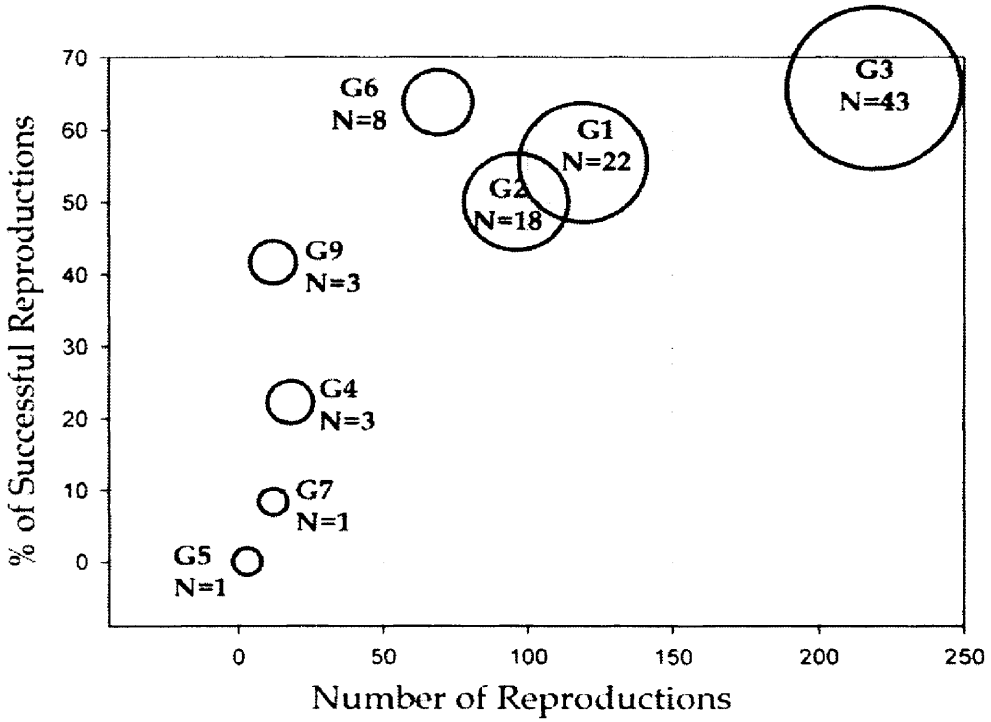

Fig 3 Proportion of successful reproduction attempts in 85 reproductive Egyptian vultures grouped according to their MHC genotypes (G1-G7, G9). Circle size is indicative of the number of individuals (N) holding each MHC genotype and the total number of reproduction observed in every case is indicated in the X axe.

Discussion

To our knowledge, our paper represents the first attempt to link neutral and functional genetic diversity to individual fitness components in a long lived threatened avian species. We observe significant HFCs at both neutral and functional markers, namely a negative relationship between neutral genetic diversity and the age of recruitment of individuals (homozygous birds recruit later) and a quantitative and qualitative effect of functional variability (MHC genes) on the breeding success of individuals and breeding pairs.

Even though it has been suggested that, in most cases, the more feasible explanation for HFCs is local effect rather than general effect (Balloux *et al.* 2004; Slate *et al.* 2004; Acevedo-Whitehouse *et al.* 2005; Luikart *et al.* 2008; Da Silva *et al.* 2009), our results on neutral loci may support the general effect hypothesis, i.e. a relationship between individual homozygosity and inbreeding. First, our population is partially inbred and hence HFCs may be generated as a result of effects of homozygosity at loci genome wide (Weir & Coskerham 1973; Hansson & Westerberg 2002; Slate *et al.* 2004). Second, the test of identity disequilibrium (ID) (recommended by Szulkin *et al.* 2010) showed a significant correlation among loci then suggesting that neutral heterozygosity is informative about genome wide diversity and therefore inbreeding (Szulkin *et al.* 2010). Third, we observed a clear correlation between neutral and functional genetic diversity which may also support averaged neutral heterozygosity as a good indicator of genome wide diversity. Nonetheless, we can not totally discard a local effect or a combination of both mechanisms given that the statistical power for detecting HFCs using 22 loci may be low (Balloux *et al.* 2004; Szulkin *et al.* 2010).

Genetic diversity and age of recruitment

Our understanding of the factors pertaining age of recruitment in long-lived species is still limited. Environmental variability has been considered as one of the main parameters regulating this behaviour, mainly through a control of the resources available for organisms, affecting energy acquisition and allocation (Erikstad *et al.* 1998; Cam *et al.* 2002; van de Pol & Verhulst 2006; Nevoux *et al.* 2007). However, within the same environmental conditions, intra population variability in reproductive performance will be determined by the heterogeneity in individual quality (Cam *et al.* 2002; van de Pol &

Verhulst 2006; Balbontin *et al.* 2007; Reed *et al.* 2008; Nevoux *et al.* 2007 and 2010; Aubry *et al.* 2009). Under the general effect hypothesis we expect that heterozygous individuals represent the higher quality birds in the population. Our results indicate that heterozygous or less inbred individuals recruit earlier (at four-five years old) than those significantly less heterozygous (recruiting from six to 10 years old). This may suggest: i) recruiting earlier is a better reproductive strategy is this species, as previously suggested for other long lived species (e.g. Oli *et al.* 2002; Blums *et al.* 2002; Charmantier *et al.* 2006; see Becker *et al.* 2007 for a review), ii) the studied population may be subjected to inbreeding depression if increase inbreeding determines the delay in the age of recruitment. We have observed that the age of recruitment in the studied insular population (the Canarian population) is on average, one year earlier (5.8 years old) with respect to its closest continental population (6.5 years old in the Iberian Peninsula in Western Europe, Grande 2006). This marked difference may indicate that divergent ecological conditions in the islands (lower inter-specific and higher intra-specific competition, environmental stability, higher availability of resources through the year) may be favouring an earlier recruitment in comparison to the continent, hence supporting directional selection to earlier recruitment in this population.

Finally, mate choice could also partially explain the observed results. It has been widely discussed the relationship between genome-wide heterozygosity or degree of inbreeding and sexual characters, then suggesting a directional preference for heterozygous mates (Reid *et al.* 2005; Kempenaers 2007; Thom *et al.* 2008, Fromhage *et al.* 2009). Even though we have not performed any analysis to test if level of inbreeding is affecting mate choice in the Canarian Egyptian vultures, theory suggest that those individuals relatively more heterozygous would be more efficient acquiring a breeding mate and then breed younger than those more inbred, which would also support our results.

Genetic diversity and breeding success

We do not find a significant relationship between neutral genetic diversity and breeding success within the Canarian population of Egyptian vulture. However, the productivity of this population is the lowest known for the species all over the world rounding half of the productivity observed in its closest continental equivalent (Donázar *et al.* 2002a). Similar relationships of productivity in insular populations versus their continental counterparts have also been described in other long-lived raptor populations (Tribault *et al.* 1992), but the causes of such low productivity in islands are widely unknown and have been included in the so called 'insular syndrome' (MacArthur & Wilson 1967; Blondel 2000). It has been previously described lower levels of genetic diversity in the Canarian Egyptian vultures compared to its continental counterpart (Agudo *et al.* 2011), therefore and whereas we have not performed an inter-population study, we may suppose that inbreeding could be playing a role in the comparatively lower insular productivity.

Our results indicate that when the individuals have acquired a territory and have started the reproduction, their success is related to specific functional genes (MHC genes). This finding supports the MHC theory which suggests that heterozygous individuals and heterozygous individuals with divergent MHC alleles will be at an advantage (Doherty & Zinkernagel 1975; Hughes & Yeager 1998). We observe that individuals with particular heterozygous genotypes (G3 and G6) present higher reproductive success. Furthermore, these two genotypes hold some of the most divergent pairs of alleles (Table 1). In addition, we detect that those birds presenting four alleles and the reproductive pairs with the higher number of different alleles succeed significantly more times than individuals with two alleles.

We have recently described, in a previous study, depauperate levels of genetic diversity at MHC genes in the Canarian population. The observed genetic diversity and allele frequencies were compatible with a scenario of insular foundation and bottleneck and we suggested a prominent role of genetic drift in shaping the observed MHC configuration. However and based on the comparison with neutral genetic diversity, we also detected that selection may have acted to favour the most beneficial allele configurations (Agudo *et al.* in press). Present results corroborate those findings and show that in fact, both heterozygous and those individuals holding certain group of alleles are being favoured since they present higher breeding success. In addition, due to the sharp increased in pathogen richness and load observed in the last decade in this insular population (Gangoso *et al.* 2009b), MHC diversity and consequently the individual capability to respond to pathogens, may be becoming the more critical feature affecting the individual fitness.

Finally, we observe that when contrasting reproduction among pairs, only the female MHC genotype is significantly correlated with success. As it is described above, pathogen load can be crucial in determining the survival of individuals and this is particularly critical for the young animals. The humoral, antibody-mediated immune system matures slowly in neonatal vertebrates, restricting them to fighting off infections and parasites with the innate immune system (Klasing & Leshchinsky 1999; Grindstaff *et al.* 2003). Hence, given that vertebrate neonates have a rudimentary immune defence early in life, the antibodies transferred from the mother to the offspring can protect it from infection and will constitute an important addition to the neonate's ability to cope with pathogens (Hasselquist & Nilsson 2009).

The whole picture: falling in the extinction vortex

Small and bottlenecked populations may undergo a gradual loss of genetic diversity and increase of inbreeding. Inbreeding may affect the individual fitness by, as in the present case, delaying the age of recruitment. The delay in the age of recruitment in populations of long-lived species will reduce the populations' growth rate therefore accelerating their risk of extinction (Weimerskirch 1992; Congdon *et al.* 1993; Saether & Bakke 2000; Eberhardt 2002; Grande *et al.* 2009). On the other hand, depauperate levels of genetic diversity at important functional genes may affect the individual fitness as well as decrease the global population productivity. Finally, reduced genetic variability may determine a lower ability to respond to the new environmental challenges arising, as for instance, the arrival of new pathogens (Agudo *et al.* in press).

Based on present results, we may hypothesize that natural selection could be favouring heterozygous individuals then helping to maintain higher levels of genetic diversity than expected for a reduced, isolated and inbreed population (Agudo *et al.* 2011). On the other hand, our results clearly indicate that the genetic deterioration in small populations has a negative impact on the individual fitness (e.g. Keller & Waller 2002; Brook *et al.* 2002; Spielman *et al.* 2004; Frankham 2005; Blomqvist *et al.* 2010) by acting, as shown here, on the individual reproductive performance. In our study case the conjunction of deterministic factors generally related to human activity (i.e. adult non natural mortality due persecution (Donázar *et al.* 2002; Gangoso *et al.* 2009a), the arrival of new pathogen species (Gangoso *et al.* 2009b; Agudo *et al.* in press)) and the effects associate to depleted levels of genetic diversity (inbreeding depression) can have catastrophic consequences in the population permanence in a mid-long term.

First Pages of Published Papers

The old constructions made only by stone, are camouflaged in the arid landscape of Fuerteventura. In the background, the volcano named Gairía stands and its crater, like a thirsty mouth, opens to a spring sky full of promises of rain. In the crater, the same pair of Egyptian vultures breeds since, at least, 1998.

Las antiguas construcciones majoreras hechas de piedra seca, se camuflan en el paisaje árido de Fuerteventura. Al fondo, la caldera de Gairía se yergue y su cráter, como una boca sedienta, se abre hacía un cielo primaveral cargado de promesas de lluvia. En el interior del cráter, la misma pareja de alimoches cría desde, al menos, 1998.

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Isolation and characterization of 18 microsatellite loci in the Egyptian vulture (*Neophron percnopterus*)

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Abstract We developed 18 new microsatellite loci for the endangered Egyptian vulture (*Neophron percnopterus*). Microsatellite loci were screened for variation in two different populations belonging to separate subspecies: the nominal *N. p. percnopterus* and the Canarian *N. p. majorensis*. Mean expected heterosygosities were respectively 0.51 and 0.46, while the mean number of alleles per locus was 4.7 and 3.9. These new markers allow further genetic studies for the endangered Canarian Egyptian Vulture.

Keywords *Neophron percnopterus* · Microsatellites · Egyptian vulture · Conservation genetics

Large avian scavengers are sharply decreasing all around the Old World, mainly due to human direct persecution and accidental poisoning and electrocution in power lines (Koenig 2006). Egyptian vultures (*Neophron percnopterus*) were continuously distributed from the Iberian Peninsula to India and from the Magreb to South Africa. At the present, the species has disappeared in most developed countries in southern Europe, Middle East and North Africa, where only small isolated populations persist (BirdLife 2007). In addition, it vanished from many islands such as Cyprus, Crete, and Malta in the Mediterranean (Levy 1996) and in most of the Canarian and Cape Verde islands in the Macaronesia (Donázar et al. 2005). The relict Canarian population is currently considered as a differentiated subspecies (*N. p. majorensis*), and it is heavily threatened by human-induced mortality. Due to its small size, it may also face the risks of inbreeding (Donázar et al 2002, Kretzmann et al. 2003). Here, we describe the isolation and characterisation of 18 microsatellites loci for conservation genetic analyses of the species. The development of species-specific DNA markers will allow the genetic characterisation of the surviving populations, the estimation of the possible levels of inbreeding, the genealogical relationships between individuals and the degree of gene flow between populations.

We constructed an enriched genomic library as described by Glenn et al. (2000). DNA extractions were performed from blood samples and approximately 10 μg of high molecular weight DNA was isolated by phenol-chloroform extraction (Sambrook et al. 1989). Simultaneous restriction-ligation of genomic DNA was carried out using the RsaI restriction enzyme and double stranded linker-adapted primers according to Hamilton et al. (1999). Ligated DNA was enriched with a biotin-labelled probe mixture consisting of $(GT)_{10}$ and $(CT)_{10}$ at 10 μM each. DNA fragments with repetitive sequences were then selectively captured by streptavidin-coated Dynabeads (Oxoid) and separated by a magnetic field. Enriched DNA was eluted in 200 μl dH_2O from the magnetic beads and concentrated by vacuum centrifugation to a final concentration of ~100 ng/μl. DNA was then reamplified by polymerase chain reaction (PCR), purified and ligated into a cloning vector using pGEM-T Easy Vector II (Promega). A total of 750 positive clones were screened and checked for inserts using ABI PRISM BigDye Terminator Cycle kit (Applied Biosystems) and resolved on an ABI 3100 Genetic Analyser (Applied Biosystems). Primer pairs for 88 potentially usable microsatellite loci were designed using the software package Primer3. Polymorphism was

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BIODIVERSITY RESEARCH

Evidence of connectivity between continental and differentiated insular populations in a highly mobile species

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ABSTRACT

Aim Genetically differentiated insular populations are candidates for independent units for conservation. However, occasional immigration to reduced island populations may occur and potentially have important consequences in their future viability and evolutionary potential. In this study, we investigate the conservation implications of population structure and connectivity of insular and continental populations of a migratory raptor as determined using genetic tools and satellite tracking.

Location Western European populations in the Iberian Peninsula and two insular populations in the Mediterranean Sea (Balearic Islands) and Atlantic Ocean (Canary Islands).

Methods We genotyped 22 microsatellite loci in 96 Egyptian vultures (*Neophron percnopterus*) from the Iberian Peninsula, 36 from Menorca (Balearic archipelago) and 242 (85% of the current population) from Fuerteventura (Canary Islands). We analysed genetic variation to estimate structure, gene flow, genetic diversity, effective size and recent demographic history of the populations. Additionally, 19 vultures were marked with satellite transmitters to track their migration routes.

Results Insular populations were genetically differentiated from those of the mainland. We detected immigration in the insular populations and within the continental counterpart. We found similar levels of genetic variability between the continent and the islands, and a bottleneck analysis indicated recent sharp population declines in both archipelagos but not on the continent.

Main conclusions Our study provides evidence that, in spite of significant differentiation, insular populations of highly mobile species may remain connected with the mainland. Conservation programmes should take into account population connectivity and integrate differentiated units of management within complex units of conservation that can best maintain processes and potential for evolutionary change.

Keywords
Connectivity, island, microsatellites, migratory vulture, population genetics, satellite tracking.

INTRODUCTION

Understanding population structure and connectivity is crucial for determining units of management for wildlife conservation programmes (Moritz, 1994; Saccheri *et al.*, 1998; Segelbacher & Storch, 2002; Schtickzelle *et al.*, 2005; Palsbøll *et al.*, 2006; Anderson *et al.*, 2009). Understanding processes structuring populations in environments comprised of discrete spatial units is especially challenging (Haila, 1990). The theory of island biogeography (MacArthur & Wilson, 1967) offers a conceptual framework for the study of differentiated entities. Islands are viewed as dynamic units where immigration and extinction rates occur as functions of island area and isolation. Island isolation is thought to be subject to different spatial

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BMC Evolutionary Biology

RESEARCH ARTICLE Open Access

The role of humans in the diversification of a threatened island raptor

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Abstract

Background: Anthropogenic habitat modifications have led to the extinction of many species and have favoured the expansion of others. Nonetheless, the possible role of humans as a diversifying force in vertebrate evolution has rarely been considered, especially for species with long generation times. We examine the influence that humans have had on the colonization and phenotypic and genetic differentiation of an insular population of a long-lived raptor species, the Egyptian vulture (*Neophron percnopterus*).

Results: The morphological comparison between the Canarian Egyptian vultures and the main and closest population in Western Europe (Iberia) indicated that insular vultures are significantly heavier (16%) and larger (about 3%) than those from Iberia. Bayesian and standard genetic analyses also showed differentiation ($F_{ST} = 0.11$, $p < 0.01$). The inference of changes in the effective size of the Canarian deme, using two likelihood-based Bayesian approaches, suggested that the establishment of this insular population took place some 2500 years ago, matching the date of human colonization. This is consistent with the lack of earlier fossils.

Conclusions: Archaeological remains show that first colonizers were Berber people from northern Africa who imported goats. This new and abundant food source could have allowed vultures to colonize, expand and adapt to the island environment. Our results suggest that anthropogenic environmental change can induce diversification and that this process may take place on an ecological time scale (less than 200 generations), even in the case of a long-lived species.

Background

The negative impact of humans on biodiversity is well known and is often referred to as 'the sixth mass extinction'. For many endangered species, humans have induced fragmentation and declines in population size that have led to strong drift in many species [e.g. [1-4]]. Species endemic to islands have paid one of the highest tolls, as shown, for instance, by the massive extinctions that followed the human colonization of the Indo-Pacific archipelagos [5]. Human colonization of islands is typically associated with habitat destruction and fragmentation, as well as with other processes such as overexploitation or introduction of exotic species and pathogens that can seriously damage species richness [6,7]. In island ecosystems above all, invasions of exotic species have been implicated as an important factor in population loss and extinction [8,9]. However, alien species may also be beneficial to some native species and act, for example, as new and abundant food resources [10,11].

The unprecedented rate of anthropogenic perturbation that has affected many regions during the last centuries may be directly or indirectly promoting changes in the selective forces acting on natural populations [12]. Consequently, human activity has become associated with evolutionary changes that occur over periods of a few hundred years, otherwise known as 'contemporary evolution' [13-15]. Several studies have reported adaptation occurring through contemporary evolution in species confronting anthropogenic environmental changes [see [16] for a review]. However, whether such anthropogenic modifications can also promote phenotypic diversification and perhaps even speciation of wild vertebrates has rarely been considered. Nonetheless, it seems unlikely that human actions would have triggered divergent evolution in vertebrate populations, especially

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Conclusiones

- 1. El estudio de la estructura poblacional y flujo génico entre las poblaciones insulares y continentales de Alimoche sugiere que las poblaciones isleñas de especies con capacidad dispersiva pueden estar conectadas a sus equivalentes continentales aun cuando se hallan producido fenómenos claros de diversificación.

- 2. El análisis de la estructura poblacional de Alimoche en la Península Ibérica sugiere la reciente fragmentación de una población Ibérica panmíctica y la consecuente diferenciación genética y aislamiento de los alimoches andaluces, los más amenazados.

- 3. A pesar del progresivo declive que están sufriendo todas las poblaciones de Alimoche, los niveles de diversidad genética de la especie nos son alarmantemente reducidos y por el contrario, comparables a los de otras especies similares en mejor estado de conservación. Por otro lado, cabe destacar la similitud encontrada entre los niveles de diversidad genética insular y continental, probablemente facilitada por la existencia de cierto flujo génico.

- 4. Respecto a la diversidad observada en los genes del Complejo Mayor de Histocompatibilidad (MHC) clase II B, las poblaciones insulares de Alimoche (Canarias y Baleares) presentan menor diversidad genética que su equivalente continental (Península Ibérica). Los patrones observados sugieren que ha sido la deriva génica la fuerza evolutiva predominante en estas poblaciones insulares, por encima del las fuerzas de selección.

- **5.** La co-evolución de las dos copias amplificadas del gen del MHC, sugerida por nuestros resultados, determina la co-segregación de pares de alelos divergentes que se encuentra en fuerte desequilibrio de ligamiento. Dicho fenómeno ha podido contrarrestar, en parte, la pérdida de diversidad genética acontecida en las islas, al aumentar la capacidad de respuesta inmune promoviendo la co-segregación de las combinaciones de alelos más eficientes.

- **9**. La colonización y establecimiento de los alimoches canarios tiene lugar hace aproximadamente 2500 años coincidiendo con la llegada de los primeros colonizadores humanos (beréberes) y su ganado (cabras). La llegada de esta fuente abundante de alimento permitió no solo el asentamiento de esta especie en las islas si no también su explosión demográfica y consecuente diferenciación al adaptarse a nuevo ambiente insular, en un periodo de tiempo muy corto (tan solo 200 generaciones).

- **10**. Existe una correlación negativa entre la heterozigosidad individual y la edad de reclutamiento de los jóvenes en la población reproductora del alimoche canario. Así mismo, se observa una correlación negativa entre la diversidad en los genes del MHC y el éxito reproductor, y una relación cualitativa que sugiere que los individuos con determinadas combinaciones de alelos son más exitosos en la reproducción.

- **11**. El presente trabajo demuestra que el deterioro genético sufrido por las poblaciones reducidas tiene efectos negativos en la eficacia biológica individual lo que a su vez puede determinar a más largo plazo las probabilidades de supervivencia de una población.

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